Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

Generation of polyclonal and monoclonal antibodies against *Babesia ovis* protein extract

Lenira Taísse Silva Santos

DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE MESTRE EM CIÊNCIAS BIOMÉDICAS

Fevereiro, 2019

Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

Generation of polyclonal and monoclonal antibodies against *Babesia ovis* protein extract

Autor: Lenira Taísse Silva Santos

Orientador: Doutora Sandra Isabel da Conceição Antunes (IHMT, UNL)

Coorientador: Doutora Ana Isabel Amaro Gonçalves Domingos (IHMT, UNL)

Dissertação apresentada para cumprimento dos requisitos necessários à obtenção do grau de mestre em ciências biomédicas.

Dedico esta tese à minha mãezinha…

Foi e sempre será um exemplo de força e coragem!

Acknowledgments

Foram muitas as pessoas que foram estando ao meu lado neste desafio…

Em primeiro lugar quero agradecer à minha orientadora, Doutora Sandra Antunes, por toda a confiança que depositou em mim. Por todo o apoio e suporte. Por estar lá sempre que eu precisei…. Obrigada pelo seu rigor, aliado à leveza e simpatia!

Queria também agradecer à Doutora Ana Domingos, minha coorientadora que sempre mostrou toda a disponibilidade para tudo o que fosse necessário durante este desafio. Agradeço por me ter integrado no seu espetacular grupo científico, trabalhador e ao mesmo tempo com uma simpatia imensurável. Agradeço todos os dias por isso!

A todo o grupo da professora Ana Domingos, prestando especial ênfase à Joana Couto, uma "menina" muito interessada sempre pronta a ajudar qualquer um. Admiro muito a tua capacidade de bem receber, e ajudar todos os que vão passando pelo grupo. OBRIGADA Joana…

À Catarina Rosa, que quando cheguei ao grupo era na altura, igualmente estudante de mestrado e trabalhadora-estudante, provando que também se conseguia com toda a primazia e principalmente um obrigado por me transmitires tanto amor pela cultura de *Babesia ovis*, que viria a ser necessário.

À Carolina que foi a grande impulsionadora deste desafio… e que com muita alegria sempre foi dando muita força.

A todos os professores e colegas de mestrado do IHMT, com que fui partilhando uma parte desta experiência.

Aos meus pais deixo um profundo agradecimento. Sei que batalharam muito para alcançar tudo aquilo que alcançaram. O resultado traduz-se nas pessoas que eu e o meu irmão Cláudio nos tornámos e disso não tenho dúvidas. À minha mãezinha, minha estrela, que certamente estará a olhar por nós e terá orgulho na "batalha" que travei. Ao meu pai, obrigada por seres o primeiro a dizer que o estudo nunca é demais e, portanto, todo o apoio me deste nesta decisão.

Ao meu irmão Cláudio, por ser um exemplo de luta para alcançar aquilo que realmente amamos. Desejo profundamente que concretizes o teu desejo manino… estás sempre mais próximo e tudo farei para te ajudar nessa conquista. Tu Sabes!

À Jéssica e ao irmão, obrigada por ter uns sobrinhos tão lindos e amorosos, que tanto alento trouxeram, e principalmente ao sentido que deram à vida (que por momentos nos trouxe tanta revolta e angústia).

À Alexandra, à Rita e Andreia, minhas amigas de sempre. Mesmo que fiquemos 3 mil anos sem falar, quando falamos é sempre igual!

À Graça e à Cesaltina, que são prova que um bom amigo será sempre um bom remédio! Vocês deviam ser donas de uma “Farmácia”!

A todos os meus colegas de trabalho que indiretamente me ajudaram neste desafio…

A ti, Sérgio…. Thank you for supporting me in this journey since our first day!!

Resumo

Produção de anticorpos policlonais e monoclonais contra um extrato proteico de *Babesia ovis*

Lenira Taísse Silva Santos

Palavras-chave: *Babesia ovis*, hibridomas, anticorpos policlonais/monoclonais, extrato proteico, péptido PCCA, *Rhipicephalus bursa*.

A babesiose é uma doença com importância significativa na saúde animal e humana, com impacto socioeconómico mundial, causada pela invasão/infeção de células eritrocitárias por protozoários do género *Babesia*, dentro do qual são conhecidas mais de uma centena de espécies. Dentro deste grupo de organismos, algumas espécies são reconhecidamente capazes de infetar humanos, sendo os animais domésticos ou silváticos o seu reservatório, fazendo desta doença uma zoonose. Nos últimos anos têm sido reportados diversos casos de babesiose humana, mas o impacto mais significativo desta infeção acontece na área veterinária uma vez que estes agentes são transmitidos por ixodídeos durante a sua alimentação sanguínea no hospedeiro. A prevalência desta doença está intimamente ligada à distribuição dos ixodídeos, uma vez que os agentes etiológicos apresentam uma certa exclusividade de vetores e hospedeiros vertebrados.

A *Babesia ovis* é uma das espécies que causa babesiose ovina, responsável por elevada mortalidade e morbilidade de animais infetados, contribuindo anualmente para elevadas perdas económicas. Sendo transmitida maioritariamente pela carraça *Rhipicephalus bursa* a sua distribuição sobrepõe-se à do vetor que se encontra na região mediterrânea. Sendo que não existe vigilância ativa desta doença a sua prevalência está certamente subestimada. O controlo desta e outras babesioses pode ser direcionado, quer ao agente etiológico, quer à carraça-vetor que o transmite. No entanto, as escolhas são limitadas a fármacos anti-*Babesia* e vacinas atenuadas, no primeiro caso e acaricidas no segundo. Estas questões suportam não só a necessidade de uma vigilância epidemiológica, bem como o progresso na descoberta de novos fármacos anti-*Babesia*. Desta forma o atual estudo foca o desenvolvimento de um método de diagnóstico serológico fazendo uso da tecnologia de produção de anticorpos poli/monoclonais. Para tal, foi utilizado um extrato de proteínas de *B. ovis*. Sendo o parasita intraeritrocitário, passou-se pelo desafio de otimizar um protocolo de isolamento dos merozoítos presentes na cultura *in vitro* de *B. ovis*, de forma a obter um extrato livre de proteínas das células eritrocitárias. Paralelamente, foi também utilizado um peptídeo de uma proteína de *R. bursa*, Propionyl-CoA carboxylase para validação da técnica de produção hibridomas.

A produção de anticorpos policlonais e monoclonais contra *B. ovis* representa um avanço no diagnóstico rápido da doença, que se propaga silenciosamente, com implicações no desenvolvimento também de ferramentas de controlo desta parasita.

Abstract

Generation of polyclonal and monoclonal antibodies against *Babesia ovis* protein extract

Lenira Taísse Silva Santos

Keywords: *Babesia ovis*, hybridomas, polyclonal/monoclonal antibodies, protein extract, PCCA peptide, *Rhipicephalus bursa.*

Babesiosis is a disease of significant importance in animal and human health, with a global socio-economic impact, caused by the invasion/infection of erythrocyte cells by protozoa of the genus *Babesia*, within which more than a hundred species are known. Some species, within this group of organisms, are capable of infecting humans, being domestic or sylvatic animal their reservoirs, making this a zoonotic disease. In recent years, several cases of human babesiosis have been reported, but the most significant impact of this infection occurs in the veterinary area, since these agents are transmitted by ticks during blood feeding in the host. The prevalence of this disease is closely linked to the distribution of ticks due to some exclusivity of vector and vertebrate hosts, by the etiological agent.

Babesia ovis is one of the species that causes sheep babesiosis, responsible for high mortality and morbidity of infected animals thus, contributing to high economic losses annually. Being transmitted mainly by *Rhipicephalus bursa* tick, its distribution overlaps with that of the vector found in the Mediterranean region. Since there is no active surveillance of this disease, its prevalence is certainly underestimated. The control of this and other babesiosis can be directed both to the etiological agent and the vector tick that transmits it, but the choices are limited. Anti-*Babesia* drugs and attenuated vaccines can be used in the first case, and acaricides when targeting the vector. These issues support not only the need for epidemiological surveillance but also progress in the discovery of new anti-*Babesia* drugs. Thus, the current study focuses on the development of a serological diagnostic method using poly/monoclonal antibody production technology. For this purpose, a protein extract of *B. ovis* was used. Since the parasite is intracellular, the challenge of optimizing a protocol of isolation of merozoites present in the *in vitro* culture of *B. ovis* (in order to obtain a protein-free extract from erythrocyte cells) needed to be overpassed. In parallel, a peptide from a *R. bursa* protein, Propionyl-CoA carboxylase, was also used for hybridoma generation to validate the technique.

The production of polyclonal and monoclonal antibodies against *B. ovis* represents an advance in the rapid diagnosis of the disease, which propagates silently, with implications for the development of control tools against this parasite.

Abbreviations

AP	Alkaline phosphatase
APC'S	Antigen-presenting cells
B cells	Lymphocyte B
B. ovis	*Babesia ovis*
BOPE	*Babesia ovis* protein extract
CD28	Cluster differentiation 28
CD4	Cluster differentiation 4
CD8	Cluster differentiation 8
CD80	Cluster differentiation 80
CD86	Cluster differentiation 86
DAPI	4′, 6′-diamidino-2-phenylindole
DMEM	Dulbecco´d Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	*Deoxyribonucleic Acid*
ELISA	Enzyme-Linked Immunosorbent Assay
F	Fragment
FACS	Flow cytometry
FBS	Fetal Bovine serum
Fc	Constant fragment
FITC	Fluorescein isothiocyanate
Fv	Variable fragment
GFP	Green Fluorescent Protein
H	Heavy
HAT	Hypoxanthine aminopterin thymidine
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase mutant
IFA	Immunofluorescent assay
IFAT	Immunofluorescent antibody assay
Ig	Immunoglobin
IgA	Immunoglobin A
IgD	Immunoglobin D
IgE	Immunoglobin E
IgG	Immunoglobin G

IgM	Immunoglobin M
IMDP	Diminazene aceturate and imidocarb dipropionate
iRBC	Infected red blood cell
L	Light
mAbs	Monoclonal antibodies
MHC	Major histocompatibility complex
min	Minute
pAbs	Polyclonal antibodies
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween 20
PCCA	Propionyl-CoA carboxylase
PCR	Polymerase chain reaction
PEG	Polyethylene Glycol
PVDF	Polyvinylidene fluoride
RBC	Red blood cell
RPM	Rotation per minute
SDS- PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Spp.	Species
T cells	Lymphocyte T
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with tween 20
TCR	Lymphocyte T cell receptor
TES	*N*-Tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid
v/v	volume/volume
VYMS	Vega y Martinez solution
w/v	weigh/volume
WB	Western blotting

Table of contents

Index of Figures

Index of Tables

Introduction

1.1. Aims of the Master project

Babesiosis affects a wide range of wild, domestic animals and humans. Recently, reports of zoonotic *Babesia* infection has been increasing, (Yabsley & Shock, 2013) (Vannier, Diuk-Wasser, Mamoun, & Krause, 2015) (Jia, et al., 2018) demonstrating the need for an active surveillance of this poorly studied disease, as well as progress in the discovery of novel anti-*Babesia* drugs. It would be important to develop new diagnostic methods in the future. Faster and more sensitive methods for disease diagnoses are important to be developed in the future to quickly start treatment and better control the disease. The production of monoclonal antibodies may be an approach for the creation of a new diagnostic method, a potential tool for the development of a fast and inexpensive test. Therefore, the present work aims to produce monoclonal antibodies against *Babesia ovis*, using hybridoma technology. In parallel, monoclonal antibodies against a Propionyl-CoA carboxylase peptide, from a protein which is present in the tick vector *Rhipicephalus bursa* will be produced to validate the technique.

1.2. The genus *Babesia* and babesiosis

Babesia spp. are tick-transmitted protozoan hemoparasites, with pronounced economic, veterinary and human impact worldwide, being the second most commonly found parasites in the blood of mammals after trypanosomes (Schnittger, Rodriguez, Florin-Christensen, & Morrison, 2012). Belonging to the genus *Babesia*, phylum Apicomplexa, order Piroplasmida, these organisms are responsible for babesiosis, a disease with worldwide distribution, intimately linked to the distribution of vectors (Homer, Aguilar-Delfin, Telford III, Krause, & Persing, 2000).

Babesia was named in honor to the Romanian scientist Victor Babes who was the first to discover (in 1888) microorganisms inside bovine erythrocytes of cattle that presented hemoglobinuria. Later, in 1892, he observed a similar organism in sheep blood. Five years later in the USA, Smith and Kilbour described the presence of an intraerythrocytic parasite that was the cause of tick-transmitted Texas Cattle Fever, a disease that had long afflicted cattle ranchers in the Southern US states (Schnittger, Rodriguez, Florin-Christensen, & Morrison, 2012).

Babesia spp. great evolutionary success can be proven by the description of more than hundred species that cause infection in a wide range of hosts. This parasite is

phylogenetically close to *Plasmodium* spp. and sharing many biological features such as being vector-borne protozoa transmitted by an arthropod, their life cycle including asexual multiplication in vertebrate blood cells, sexual reproduction in gut and the production of sporozoites in the salivary glands of the vector (Chauvin, Moreau, Bonnet, & Plantard, 2009).

Babesia spp. need two host to complete its life cycle, the tick vector and vertebrate host, representing a complex system of interactions (Antunes et al, 2017). The prolonged period of feeding of ixodid ticks that can last from 2 days to about 2 weeks, depending on the stage (larva, nymph or adult female) and tick species, allows an extended interaction between the tick and vertebrate host and promotes efficient *Babesia* spp. transmission (Chauvin, Moreau, Bonnet, & Plantard, 2009). Maintenance and persistence within the tick vector is ensured by transovarial and transstadial transmission, sometimes over several tick generations, depending on the developmental duration and length of questing periods of the tick (Chauvin, Moreau, Bonnet, & Plantard, 2009). After being introduced in a sporozoite form, mixed with tick salivary components, *Babesia* may then persist asymptomatically within its host for several years and can be afterwards infect a new susceptible vector during feeding, perpetuating parasite survival (Antunes et al, 2017).

Babesia ovis, B. motasi and *B. crassa* are the etiological agents of ovine babesiosis, the most important haemoparasitic tick-borne disease of small ruminants in tropical and subtropical areas of the world, which causes animal mortality and morbidity, and, consequently, high economical losses annually (Sadeghi Dehkordi, et al., 2010). The *Rhipicephalus bursa* tick, known by infesting ruminants and occasionally parasite other animals such as wild ungulates and small mammals, is the main and dominant vector of *B. ovis* (Sadeghi Dehkordi, et al., 2010). In a minor scale *R. bursa* can transmit other pathogens such as *Rickettsia* spp. and *Anaplasma* spp. (Antunes, et al., 2018).

1.2.1. Overview of the *Babesia* spp. life cycle

Remarkable differences have been documented regarding *Babesia* spp. development, what can be attributed to the variety of the species and the large variety of both vertebrate hosts and vectors. However, a general life cycle of *Babesia* species is widely accepted (Figure 1). Noteworthy, one striking difference between species is the

transovarial transmission, which occurs in *Babesia* sensu stricto and does not in other *Babesia* sensu lato lineages (Homer, Aguilar-Delfin, Telford III, Krause, & Persing, 2000). In the same way as other Apicomplexa, *Babesia* presents three stages of reproduction: (a) **gamogony** - formation and fusion of gametes inside the tick gut; (b) **sporogony**— asexual reproduction in thick salivary glands; (c) **merogony**— asexual reproduction in the vertebrate host (Saad, Khan, Ali, & Akbar, 2015).

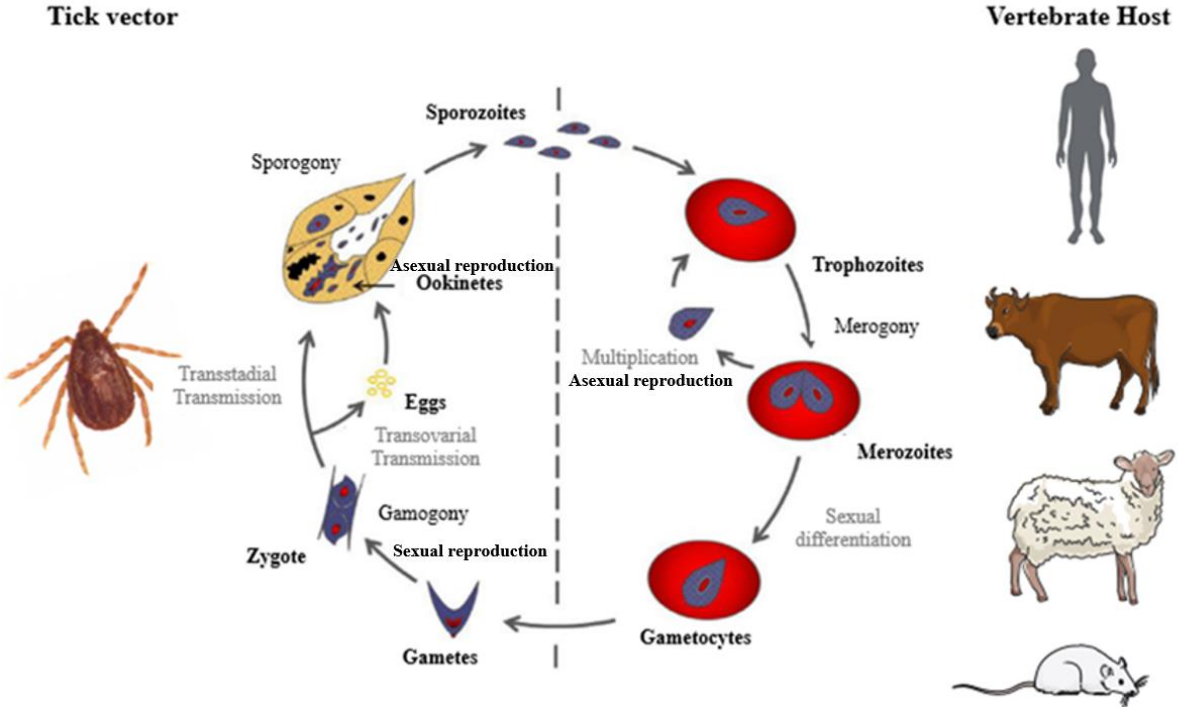

Figure 1: Overview of a generic *Babesia* spp. life cycle.
During blood meal, ticks ingest the parasites (gametocytes), from an infected host. In the susceptible tick midgut, they undergo sexual development into mature gametocytes and gametes, whose fusion results in zygotes, able to invade midgut cells. These zygotes go through meiosis and differentiate into motile prolonged kinetes, ookinetes, (gamogony) that escape midgut cells and spread to different tissues throughout the hemolymph, including the salivary glands. Here, kinetes undergo a cycle of asexual multiplication originating sporozoites (sporogony) that will infect a naïve vertebrate host during feeding. The infection is maintained throughout tick developmental stages (transstadial transmission). For *Babesia sensu stricto*, kinetes invade tick ovaries and eggs resulting in infected larvae (transovarial transmission). In the vertebrate host, upon erythrocytes invasion, parasites reproduce asexually (merogony). Adapted from: Schnittger et al. (Schnittger, Rodriguez, Florin-Christensen, & Morrison, 2012).

1.2.2. Events in the vertebrate

In *Babesia* species, after a tick bite, the transmitted sporozoites infect the vertebrate erythrocytes reproducing asexually within the erythrocytes (merogony) and are referred as piroplasms or piroplasmids due to their pear-shaped appearance, sharing the term with *Theileria*, a phylogenetically related protozoan (Jalovecka, Hajdusek,

Sojka, Kopacek, & Malandrin, 2018). Parasite orientation and penetration is mediated by proteins secreted from the apical secretory organelles through a process of invagination forming a parasitophorous vacuole. The vacuole membrane gradually disintegrates, and the parasite is left with the defining piroplasm feature of a single membrane. Inside of host erythrocytes, most merozoites become trophozoites and divide by binary fission producing more merozoites, which lyse the cell and disseminate. Destruction of host cells due to the rapid and multiple formation of parasites inside the same cells causes in the vertebrate host hemoglobinuria. The merogony is asynchronous and thus trophozoites and merozoites occur in the bloodstream simultaneously. The size of merozoites varies according to species as well as the vertebrate host species (Jalovecka et al, 2018). Some trophozoites can, however, become potential gametocytes that when they are in the gut of the tick, these gametocytes will develop into gametes prior to leaving the erythrocytes within the tick gut (Homer, Aguilar-Delfin, Telford III, Krause, & Persing, 2000).

1.2.3. Events in the tick

It is established that the *Babesia* spp. sexual phase, gamogony, occurs inside its vector, however, gametocytes, which are the first sexual stages appear in the host red blood cells. These gametocytes are predetermined to differentiate into gametes in the lumen of the tick gut mediating the ability to infect the tick vector (Jalovecka et al, 2018). Ingested *Babesia*-infected erythrocytes reach the tick midgut, where pathogen will leave the erythrocytes. During this stage asexual intra-erythrocytic stages will be destroyed or degenerate, but a small number of gametocytes change its structure resulting in the formation of gametes in which a distinctive arrowhead-shaped organelle is formed, a unique characteristic among apicomplexan parasites (Jalovecka et al, 2018). This is thought to be essential in the zygote fusion, and also in the penetration of the midgut peritrophic membrane (Chauvin, Moreau, Bonnet, & Plantard, 2009) (Gough, Jorgensen, & Kemp, 1998) (Maeda, et al., 2016) (Sonenshine & Hynes, 2008). Unlike the normally growing and asexually reproducing merozoites, the gametocytes do not reproduce (Jalovecka et al, 2018). The zygote is a motile stage capable to penetrate the peritrophic matrix and invade gut epithelial cells. Inside the epithelial cell, the zygote turns into a spherical shape undergoes a meiotic division resulting in the formation of kinetes, which are afterwards released into the tick haemolymph, reaching all tick

internal tissues. The kinetes undergo the second asexual multiplication and subsequently, invade the salivary glands, undergo sporogony, the maturation of sporozoites, (reviewed by Jalovecka et al, 2018). In the salivary glands, sporozoite development can be divided into three stages; First, the parasite expands and fills the hypertrophied host cell, forming a multinucleate sporoblast. The second step starts only after the tick host begins feeding again; the specialized organelles (micronemes, rhoptries, and double membrane segments beneath the plasma membrane) of the future sporozoites develop, finally, in mature sporozoites. Approximately 5,000 to 10,000 sporozoites can be produced within a single sporoblast. The efficiency of tick transmission is related to the tick saliva, which probably facilitates infection with its anti-inflammatory and/or immunosuppressive pharmacological activity (Homer, Aguilar-Delfin, Telford III, Krause, & Persing, 2000; Saad, Khan, Ali, & Akbar, 2015)

As previously mentioned, some *Babesia* species, namely from the *Babesia* sensu stricto lineage, possess transovarial transmission, a unique ability among all apicomplexan parasites. Ovarian cells infection and transmission via larval progeny to tick larvae occurs after the zygotes entered the haemolymph (Homer, Aguilar-Delfin, Telford III, Krause, & Persing, 2000; Saad, Khan, Ali, & Akbar, 2015).

1.2.4. Babesiosis diagnosis and treatment

For the diagnosis of babesiosis various methods may be employed, such as, microscopic detection, culture, serological tests, and molecular techniques where microscopy and polymerase chain reaction (PCR) are the most commonly elected methods (Parija & Venugopal, 2015). For microscopy, thin blood smears are stained with Giemsa, in which organisms appear as dark stained ring like with light blue cytoplasm. According to species, different forms of different sizes can be seen, ranging from simple rings (annular), paired or pyriform, single pear shaped trophozoites and rarely in Maltese cross (tetrad form) (Figure 2). Mild infections can be missed using this technique, remaining untreated, thus more sensitive molecular techniques are required such as PCR. In this technique, *Babesia* spp. DNA is targeted for amplification and when positive, the nucleotide sequence is analyzed and compared to sequences available at public databases to identify the infecting parasite. DNA presence is an indication of

an active infection, but infection can be below the limit of PCR (Saad, Khan, Ali, & Akbar, 2015). Serological tests exist as well but have some limitations in confirming active or persistent disease and indistinguishing current and previous infection, when parasite is below the limit of PCR detection (Sevinc, Cao, Xuan, Sevinc, & Ceylan, 2015). Serological testing with the indirect fluorescent antibody test (IFAT) is the most commonly used method for diagnosing chronic *B. ovis* infections (Sevinc, Cao, Xuan, Sevinc, & Ceylan, 2015). However, this type of tests, in the same way as light microscopy, requires experience, is time consuming and its results are difficult to compare from different laboratories. An enzyme-linked immunosorbent assay (ELISA) using synthetically derived *Babesia bovis* antigen has been used to detect anti-*B. bovis* antibodies in some research, however immunoreactive recombinant proteins despite widely used in serological assays for the diagnosis of equine, bovine, and canine babesiosis, there has been no report of the production of immunoreactive recombinant proteins of *B. ovis*. (Sevinc, Cao, Xuan, Sevinc, & Ceylan, 2015).

Most of the drugs available nowadays, in treatment for *Babesia* infections have problems due to toxicity and development of resistant parasites. Currently, diminazene aceturate and imidocarb dipropionate (IMDP) are the most widely used drugs for treatment of babesiosis (Rashid, Khan, Rasheed, Maqbool, & Iqbal, 2010).

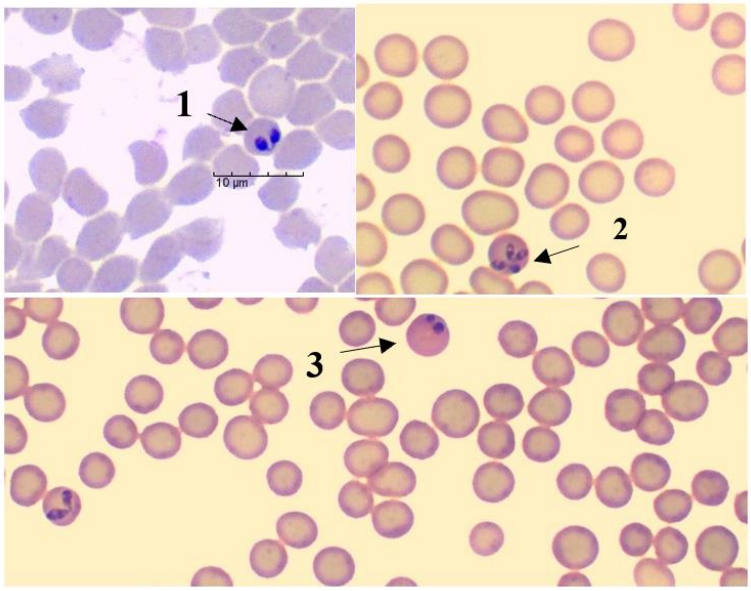

Figure 2: *Babesia ovis* forms, *in vitro* cultures
1. Merozoite characterized by piriform shapes forming a pair. **2.** Merozoite characterized by piriform shapes forming a tetrad ("Maltese cross" pattern). **3.** Single round trophozoite. Intraerythrocytic parasites were observed under a 100x amplification of a Motic BA210 LED trinocular compound microscope (original). Scale bar 10µm. Original and authorized by Catarina Rosa.

1.3. Development of novel diagnosis tools: monoclonal antibodies

1.3.1. The immune response

Immunology as a science can be traced back to 1798 when Edward Jenner proved that it was possible to protect a person from smallpox by inoculation with a similar pox, a process which was after confirmed in 1870 by Louis Pasteur and Robert Koch (Rocha & Roncato Duarte, 2011). Using antibodies as therapeutic agents was a concept introduced by Emil Adolf von Behring in 1890. When it was demonstrated that immunity can be transferred from one animal to another via serum. In his work, he managed to make healthy animals, immune to diphtheria and tetanus by using small amounts of toxins from infected animals to produce transferable immunity – earning him the Nobel prize in 1901.

All living organisms are constantly exposed to a panoply of agents capable of injure. Thus, most organisms present many lines of defense against invading foreign substances. In animals, the first line of defense against invaders consists of physical and chemicals barriers such as skin and mucosal membranes of the digestive, respiratory and reproductive tracts. The innate immune system is classically portrayed as the first and more primitive line of defense once an invader has broken through the physical barriers of the organism, taking no longer than minutes to hours to be fully activated (Cerny & Ilja, 2019). The innate immune system includes neutrophils, macrophages, natural killer cells by ancient humoral systems such as defensins and complement that act together to respond quickly and strongly to invasion (Farber, Netea, Radbrunch, Rajewsky, & Zinkernagel, 2016; Netea, Schlitzer, Placek, Joosten, & Schultze, 2019; Cerny & Ilja, 2019). This immune response many times suffices but when facing a high number of foreign substances/pathogens or high virulence of invading pathogens this initial response fails, infection is established, and the organism becomes compromised. Vertebrate animals have a third level of defense, a more refined system, with most importance to the present research, which is the adaptive immune system that can recognize and destroy specific substances. Any substance capable of generating such a response is called an antigen, or immunogen. Antigens are substances, such as toxins or enzymes, in microorganisms or tissues, foreign to the immune system. This system is characterized by specificity and immunologic memory, which is the ability of the adaptive immune system to mount a stronger and more effective immune response against an antigen after a first encounter with that antigen. (Netea, Schlitzer, Placek,

Joosten, & Schultze, 2019) (Farber, Netea, Radbrunch, Rajewsky, & Zinkernagel, 2016) Establishment of the adaptive immunity needs 1–2 weeks to be mounted but is crucial in latter phases of infection and secondary infections. First regarded as compartmentalized responses in time, the innate and adaptive immune processes are now recognized as intertwined and coordinated responses (Netea, Schlitzer, Placek, Joosten, & Schultze, 2019).

1.3.2. The adaptive immune system: lymphocytes function in the immune response

The adaptive immune system uses a complex system of signaling between the lymphocytes and either an array of specialized cells or the complement system to deal with foreign antigens (Figure 3). Lymphocytes are responsible for the specificity of adaptive immune responses, divided in B cells and T cells which names derive from the organ where they origin (bone marrow and thymus). Nonactivated lymphocytes are similar but once activated by antigen, proliferate and mature into effector cells, where B cells secrete antibodies unlike T cells. These can be divided in two classes, cytotoxic and helper T cells. Cytotoxic T cells kill infected cells, while helper T cells help activate macrophages, B cells, and cytotoxic T cells. (Alberts, Johnson, & Lewis, 2002). Some cells of the innate immune system act as antigen-presenting cells (APCs), presenting antigens to T cells, triggering an adaptive immune response.

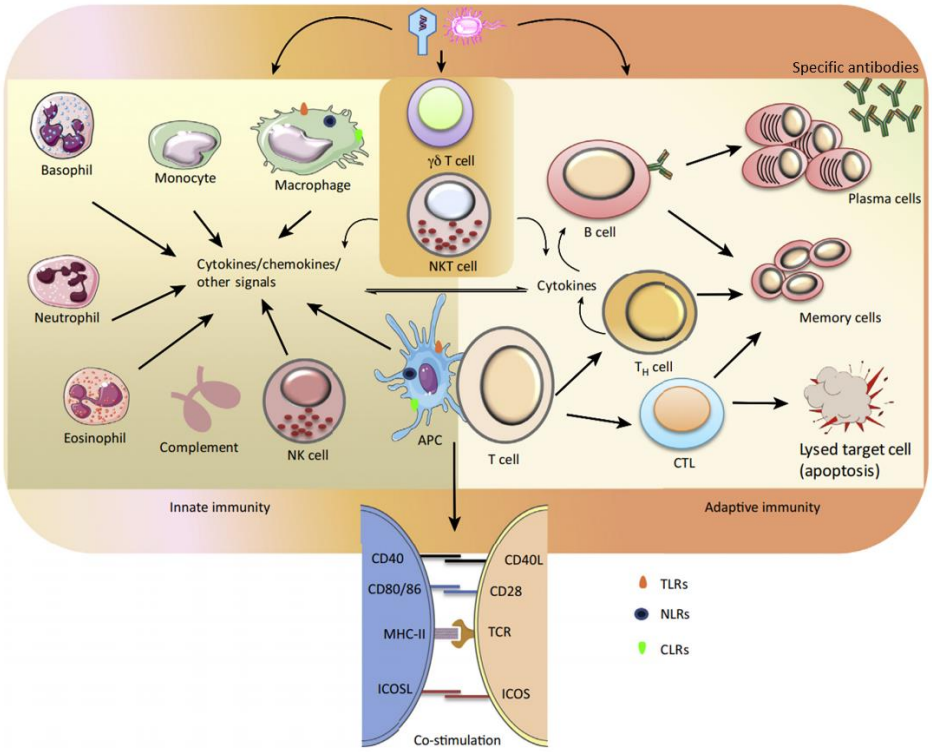

Figure 3: Representation of innate and adaptive immune systems
The innate immune system constitutes a front line of defense and provides a nonspecific response against invading pathogens. This response is mediated by various cells (granulocytes, monocytes, macrophages, dendritic cells, neutrophils, basophils, and natural killer cells, and active molecules as proteins of the complement cascade) through recognition by pattern recognition receptors. The innate immune response shapes adaptive immunity resulting in the production of antigen-specific T and B lymphocytes. Adapted from Bonam et. al. (Bonam, Partidos, Halmuthur, & Muller, 2017).

1.3.3. B cell and T cells function in the immune response

T cells are programmed to be specific for an antigen, once they leave the thymus, they circulate throughout the body until they recognize their antigen on the surface of antigen presenting cells. The T cell receptor (TCR) on both $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells binds to the antigen as it is performed in a major histocompatibility complex (MHC), on the surface of the antigen-presenting cells (APC), starting activation of the T cells. The CD4 and CD8 molecules then bind to the MHC molecule too, establishing the whole structure. This initial binding between a T cell specific for one antigen and the antigen-MHC, that normally takes place in the secondary lymphoid organs, sets the whole response in motion, that will require a secondary signal to become activated and respond to the threat. Helper T cells provided by CD28 molecule, bind to one of two molecules on the APC – CD80 or CD86 – and

initiates T-cell proliferation. This process leads to the production of many millions of T cells that recognize the antigen (Alberts, Johnson, & Lewis, 2002; Chaplin, 2010; Pennock, et al., 2013). After activation, some of the T cells migrate to the site of infection, to help innate immune cells while other activated T cells aid B cells respond to the antigens. The activated B cells secrete antibodies that specifically bind to the foreign antigen (Pennock, et al., 2013). This antigen-antibody complex is taken up by the B cell and cleaved up by proteolysis into smaller peptides (Alberts, Johnson, & Lewis, 2002). The B cell displays these antigenic peptides on its surface major histocompatibility complex (MHC) class II molecules. This combination of MHC and antigen attracts a matching helper T cell, which releases lymphokines and activates the B cell (Pennock, et al., 2013). The activated B cell then begins to divide, and progeny cells secrete millions of copies of the antibody. These antigen-specific antibodies circulate in blood plasma and lymph and bind to any additional identical antigens present (Ollila & Vihinem, 2005). Subsequently, antibodies opsonize the antigen for destruction by complement activation or for uptake and destruction by neutrophils, macrophages and natural killer cells (Chaplin, 2010).

1.3.4. Antibody structure, classes and isotypes

Synthesized exclusively by B cells, antibodies defend the organism, binding and consequently inactivating viruses and microbial toxins. Collectively, antibodies are called immunoglobins (Ig) and they are the most abundant protein components in blood, constituting about 20% of total protein in plasma (Alberts, Johnson, & Lewis, 2002). All antibody molecules made by an individual B cell have the same antigen-binding site (Chaplin, 2010). The basic structural unit studies conducted by Rodney Porter and Gerald Edelman in "The chemical structure of antibodies" study, established that a typical and simplest immunoglobulin are Y-shaped molecules with two identical light (L) chains (each containing about 220 amino acids) and two identical heavy (H) chains (containing about 440 amino acids) (Alberts, Johnson, & Lewis, 2002). Characteristic Y shape of antibody is formed by two heavy chains and two light chains. A light chain pairs with a heavy chain, and two heavy chains pair up by covalent interchain disulfide bonds and noncovalent interactions to (Figure 4).

It was demonstrated by Porter that an antibody molecule can be cleaved by papain to give two Fab fragments, which bind to but do not cross-link antigens, and Fc

fragment which can bind complement after binding an antigen. On the other hand, pepsin cleaves the antibody molecule to give a bivalent F (ab') 2 fragment which can bind, and cross-link antigens and cell receptors. The hypervariable regions (VH and VL) of the antigen-binding fragment (Fab) region are specific in binding an antigen. The crystallizable fragment (Fc) region of the antibody has an effector function, and it binds the Fc receptors on effector cells, linking the humoral response to a cellular response (Figure 4).

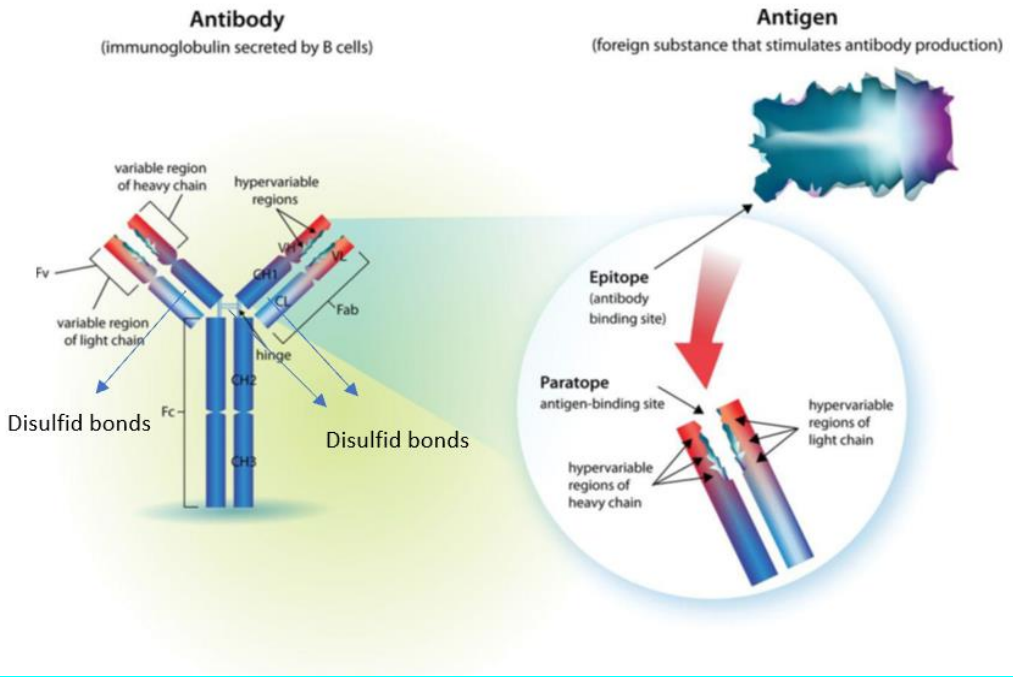

Figure 4: Illustration of an antibody structure.
CH indicates heavy chain, constant domain; CL, light chain, constant domain; Fv, variable fragment; VH, heavy chain, variable domain; and VL, light chain, variable domain. Adapted from Foltz et al (Foltz, Karow, & Wasseman, 2013)

Five isotypes of antibodies exist and are distinguished by differences in their heavy chains: α, δ, γ, ε and μ giving IgA, IgD, IgG, IgE, and IgM respectively. The IgA exists as a monomer in circulation, though usually as J chain-linked dimer. The J chains are important in the dimerization of IgA and IgM. IgA is the primary immunoglobulin in external secretions like breast milk, tears, and saliva. IgA has two subclasses; IgA1 made by bone marrow B cells and IgA2 by mucosal B cells. IgD, co-expressed with IgM on naïve B cells is secreted as a monomer with an extended hinge region and the specificity of antigen recognition is the same with IgM (Wen, et al., 2019). IgG is the most abundant immunoglobulin; it functions in the secondary phase of an immune

response, existing four subclasses of IgG (IgG1–IgG4) in humans whose abundance, half-life and binding affinity vary (Vidarsson, Dekkers, & Rispens, 2014). IgE is mainly found in the lungs and skin and is responsible for allergic reactions and has high binding affinity to mast cells and basophils. IgM is the first antibody to be secreted during an infection and mediates the primary antibody response. It is secreted as a pentamer made up of five antibodies. It plays a major role in phagocytosis by activating the complement system (Wen, et al., 2019). In the peripheral lymphatic organs, mature B cells are stimulated by antigen, and activated to proliferate and differentiate into short lived plasma cells, which can synthesize and secrete antibodies. Each plasma cell produces only one type of Ig molecule, which has a unique antigen binding specificity. During antibody production, the type of Ig produced is transformed from the IgM isotype to IgG, IgA, or IgE isotypes under the influence of helper T cells and mediated by T cell-derived cytokines (Wen, et al., 2019).

Upon entry into the body at least a few foreign antigens will die or be killed by lysozyme or preexisting antibody and complement or killed intracellularly following phagocytosis. The fragments resultant from death or degradation of foreign antigen will be finally phagocytosed and presented to T and B lymphocytes (Goding, 1996). Within 2-3 days, the first specific IgM antibodies from the primary response will be detectable. IgM is secreted as pentamers possessing ten binding sites for a fast clearance of pathogens which are often polymeric with multiple repeating motifs. Even if one antigen-combining site doesn't attach, the others will. Due to the fast response the binding is not very affine but efficient because ten antigens could be bound by one pentamer molecule. IgM is also particularly efficient at activating complement, and thus acts as an excellent primary defense (Hanack, Messerschmidt, & Listek, 2016). In 3-4 days, after affinity maturation, triggered by primary antigenic challenge, the immune cells can secrete highly affine IgGs targeting the corresponding antigen and label it for degradation. The cells producing highly affine IgGs differentiate into long living memory cells, residing in the tissues and lymph nodes and capable of a fast reactivation if an infection with the same pathogen takes place at later time points (Hanack, Messerschmidt, & Listek, 2016). Like IgM, IgG antibodies activate the complement which facilitates phagocytosis by binding to specific receptors for IgG that are presented on the surface of many phagocytic cells (Goding, 1996).

1.3.5. History of monoclonal antibodies

In 1975, monoclonal antibodies (mAbs) were first described by George Köhler, from West Germany, and Cesar Milstein from Argentina. Based on previous scientific works, Köhler and Milstein were able to fuse B cells with myeloma cell lines forming hybridoma, an immortal hybrid cell that produced antibodies against known antigens. Together with Niels K. Jerne, Köhler and Milstein shared the Nobel Prize in Physiology or Medicine in 1984 due to discovery of the principle for production of monoclonal antibodies (Köhler & Milstein, 1975; Klitgaard, et al., 2006). The discovery of mAbs deserved a major attention from several researchers, becoming a pivotal advance in medicine and triggered a series of studies on cell types. Their potential applications in diagnostic and therapeutic medicine being the focus among researchers, clinicians, and biotechnology companies (Funaro, et al., 2000).

1.3.6. Hybridoma technology

Hybridoma technology was the first method for production of monoclonal antibodies (mAbs) demonstrated (Hanack, Messerschmidt, & Listek, 2016). There are currently other methodologies to produce monoclonal antibodies, but the principle of production of these antibodies is based on the classic procedure that involves the fusion of an immortal myeloma cell with an antibody producing splenic B cell (Pandey, 2010). Significant advances have been made over the years in the design of mAbs as therapeutics; improved bioavailability, optimized affinity, improved binding specificity, and human antibody sequences to reduce any immunogenic side effects (Shukla & Thömmes, 2010). In the classic procedure, a mouse is immunized with an immunogenic compound, that has been previously purified and characterized, producing an immunological response. Afterwards, the mouse is sacrificed (when achieves satisfactory titer), the spleen is removed and the B lymphocytes present are obtained to fuse with myeloma hypoxanthine-guanine phosphoribosyl transferase mutant (HGPRT) cells. The selection of these mutant cells is made prior to fusion with addition in the culture medium of 8-azaguanine. These myeloma cells cannot synthesize nucleotides by savage pathway because they lack HGPRT. In that way the cells that possess HGPRT will incorporate these bases into the DNA and die, surviving the negative HGPRT cells. Cellular fusion is promoted by electrofusion or chemical fusion method, with PEG (Polyethylene Glycol), grown in a hypoxanthine aminopterin thymidine (HAT)

medium, that inhibits the *de novo* nucleotide biosynthesis pathway, and which makes the cells become auxotrophic for nucleic acids supplemented in the HAT media. Since myeloma cells lack the HGPRT gene, unfused myeloma cells do not grow in the HAT medium because they cannot produce DNA, dying after seven days. On the other hand, unfused spleen cells have short life span, therefore they will not grow, leaving only the hybridoma cells. A significant growth is recorded in 7-14 days after fusion. After ELISA screening, positive specific antibody-secreting hybridomas are transferred to larger culture flasks to obtain large quantities of antibodies. In a later phase, each individual clone can be separated by dilution into different culture wells. The cell culture medium can then be screened from many hundreds of different wells for the specific antibody activity required and the desired B-lymphocytes grown from the positive wells and then re-cloned and retested for activity (Köhler & Milstein, 1975; Tyagi, Sharma, Kumar, & Visht, 2011) (Figure 5).

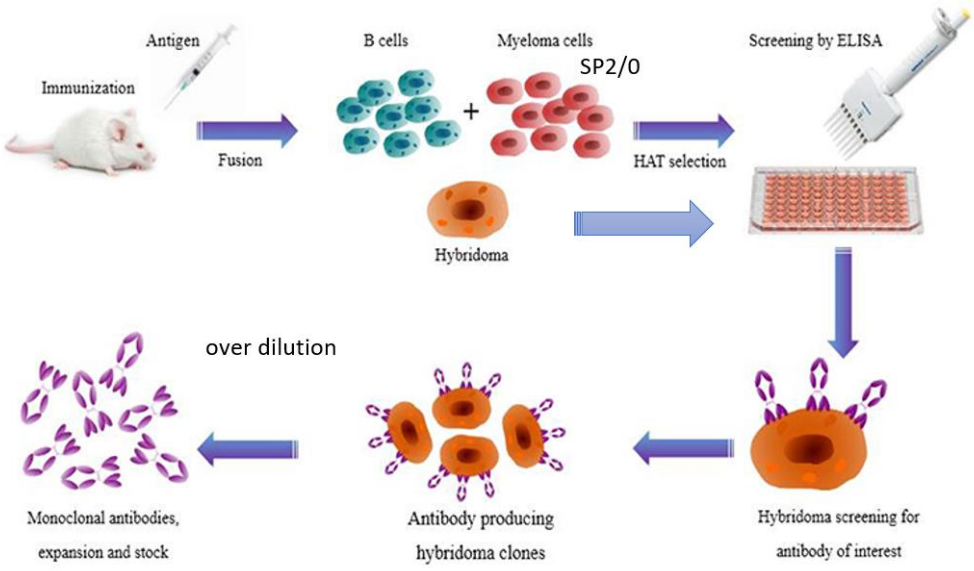

Figure 5: Schematic Representation of Hybridoma Technology.
Monoclonal antibodies are generated by immunizing laboratory animals with a target antigen. B cells and myeloma cells are fused and then selected in HAT medium. Finally, hybridoma cells producing the desired antibodies are screened. Adapted from Saeed et. al. (Saeed, Wang, Ling, & Wang, 2017).

1.3.7. Monoclonal versus polyclonal antibodies

The discovery of hybridomas antibody producing cells method (Kohler and Milstein, 1975), presented an important immunological advance. Without this discovery, polyclonal serum was the only antibody option available for scientists, which

despite being pool of antibodies produced by thousands of different B cell plasma clones and recognizing different epitopes on the same antigen, may contain antibodies specific for other antigens (such impurities in the inoculum). The polyclonal antibodies (pAbs) present the advantages of low production costs, higher affinity, a spectrum of specific binding epitopes and a mixture of antibody classes (Voskuil, 2014). Distinctively, mAbs are produced for a single B cell parent clone, recognizing only a single epitope per antigen and are less susceptible to cross-react with other proteins. B cells are immortalized by fusion with myeloma cells, allowing for long-term generation of identical mAbs (Kwakkenbos, Helden, Beaumont, & Spits, 2016). Thus, a tool to supply antibody production in large amount, which can be produced and purified with defined properties, with specificity for a single epitope, with rapid binding to antigen (Frank, 2002).

With an extensive range of applications, mAbs has been applied for diagnosing human and animal diseases, identification of molecules, viral identification, authenticity and truthfulness of genetically modified organisms (Rocha & Roncato Duarte, 2011). Importantly, mAbs have also been used in oncology, autoimmune and inflammatory therapeutics (Hanack, Messerschmidt, & Listek, 2016) (Shukla & Thömmes, 2010).

Material and Methods

2.1. *In vitro Babesia ovis* culture

B. ovis in vitro cultures were established in a biosafety level 2 facilities, following an adapted protocol from Vega *et al.* (Vega, Buening, Green, & Carson, 1985).

Commercial defibrinated lamb blood (BioRabbit, Lisbon, Portugal) was washed (described forward in 3.1.2) and maintained with Vega y Martinez solution (VYMS) (solution preparation described forward in 3.1.1) to obtain ovine red blood cells (RBCs) and stored at 4ºC for up to 5 weeks. Cryopreserved Israeli strain of *B. ovis* infected red blood cells (iRBC) at known parasitemia were used to initiate the culture (initiation described forward in 3.1.3). Parasites were cultured in 10% (vol/vol) defibrinated lamb RBC maintained in a 199 Medium (Gibco, Thermo Fischer Scientific) (1x) supplemented with Earle's Salts, L-glutamine and L-Amino acids and buffered with 25 mM HEPES. Culture complete medium was obtained by addition of 20% (vol/vol) lamb serum (Gibco, Thermo Fischer Scientific) and Antibiotic-Antimycotic (100x) (Gibco, Thermo Fischer Scientific), 1 mM L-cysteine-HCl, 2 mM bathocuproine-disulfonic acid and 25 mM *N*-Tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (all from Sigma-Aldrich). Parasite culture was maintained in a microaerophilic stationary phase atmosphere *(*5% CO_2, 2% O_2 and 93% N_2) using a hermetical incubation box at 37ºC in a CO_2 chamber. Daily, 80 % of the medium was replaced with freshly prepared complete medium and culture parasitemia was monitored by preparation blood smears stained with Hemacolor® Rapid staining of blood smear (Merk Millipore). Intraerythrocytic parasites were observed under a 100x original magnification on a Motic BA210 LED optical microscope. Every 4 days, fresh RBCs were added to the culture, performing a split or expansion. For these, the initial culture was used in a proportion of 1:2 or 1:3 of volume of initial culture to volume of medium with fresh RBCs.

When necessary *B. ovis* culture was cryopreserved using a cryoprotectant preparation (20% polyvinylpyrrolidone-40 (PVP-40) (Sigma-Aldrich) in VYMS). For this, culture was firstly centrifuged at 1300 RPM for 15 min at 4 ° C and supernatant discarded. The volume of the packed RBCs was estimated, and cryopreservation solution was added in a proportion of 1:1. After, 1 mL per cryotube was dispensed, the tube labeled with *B. ovis*, date, percentage of parasitemia and stored in liquid nitrogen.

2.1.1. Preparation of Vega y Martinez solution

Initially, 5x stock solution (VYMS 5x) was made by the addition of the following compounds: dehydrate calcium chloride ($CaCl_2.2H_2O$), potassium chloride (KCL), potassium dihydrogen phosphate (KH_2PO_4), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), di-sodium hydrogen phosphate heptahydrate ($Na_2HPO_4.7H_2O$), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$), sodium chloride (NaCl), glucose, adenine and guanosine, adjusting pH to 7.2 with 5M NaOH solution. Finally, the solution was filtered with a sterile filtration unit of 0.22 µm, at a sterile flow hood. A working solution (VYMS 1x) was prepared in a sterile flow hood, by adding one part of VYMS 5x to four parts of MilliQ H_2O. This solution was also filtered with sterile filtration unit (0.22 µm). Both solutions were stored at 4ºC.

2.1.2. Preparation of packed RBCs

Commercial defibrinated lamb blood (BioRabbit, Lisbon, Portugal), was used to maintain *B. ovis*. Once received the blood was washed before using in parasite culture as follows. The blood was divided in two 50 mL falcons and centrifuged during 20 min at 2500 RPM at 4ºC. The plasma was discarded and VYMS 1x was added reaching the final volume of 50 mL x and then centrifuged during 20 min at 2500 RPM at 4ºC. This process was repeated until a clear supernatant was obtained. Packed RBCs were stored at 4ºC for up to 5 weeks.

2.1.3. Initiation of a *Babesia ovis* culture

For each cryotube (1 mL) used, 48 mL of culture complete medium was prepared. Each cryotube was thawed, by placing it inside a goblet filled with warm water (37ºC) and agitated. When the cryotube content started to melt, it was transferred to a 50 mL falcon filled with 48 mL of culture complete medium. The solution was centrifuged at 1300 RPM during 15min at 4ºC. After centrifugation, the supernatant was carefully aspirated, discarded and replaced with 2 mL of a suspension of complete medium with RBCs. This suspension, one ml per well, was placed in a 24 wells culture plate (Corning Costar®, New York, USA) and incubated at 37ºC in a 5% CO_2, 2% O_2 and 93% N_2 atmosphere.

2.1.4. Determination of culture parasitemia

The culture viability and culture parasitemia were controlled daily. For thisa smear with packed RBCs from the culture was prepared and stained using Haemacolor®. The parasite presence was observed in microscope using the immersion oil objective 100x under a Motic BA210 LED optical microscope. At least 1000 RBCs were counted, and the number of infected and uninfected RBCs was annotated. The parasitemia was calculated as it follows:

Number of infected RBC ---------- total number of RBC

X ---------- 100

2.1.5. Flow cytometry to detect and quantify *Babesia ovis* in cells

Light microscopic manual count is the current gold standard for parasite quantification. The ability to determine parasite density in whole blood is crucial to understand disease pathogenesis and finding a suitable automated method of *Babesia* parasite quantification would facilitate higher throughput and provide results that are more objective. Flow cytometry assay is a very useful technique helping to determine parasitemia or confirmation of parasitemia compared to existing conventional techniques for this type of study, which require a lot of practice and a is a time consuming. Moreover it has been established as a reliable and precise for the measurement of parasitemia of intracellular parasites, thus a practical stain protocol was performed similar to what has previously been described (Jang, et al., 2014; Rossouw, et al., 2015; de Villiers, Quan, Troskie, Jordaan, & Leisewitz, 2019) with SYBR Green I Invitrogen (Sigma) nucleic acid gel stain—10,000× concentrate in dimethyl sulfoxide with FACS determination of parasitemia in a *B. ovis* culture was developed. Briefly, a *B. ovis* culture (>6% parasitemia) in 10% of RBCs was serially diluted by twofold with uninfected RBCs to obtain parasitemia ranging from 0.001 to 6%. First, 2 mL of *B. ovis* culture in a 15 mL Falcon was washed with PBS, for that, 2 mL of PBS was added and centrifuged at 3500 RPM for 10 min discarding the supernatant. This process was repeated three times, to eliminate debris. After washes, the suspension was turned into

1-2,5% hematocrit by adding 198 µL of PBS to a 2 µL of erythrocyte concentrate. In an ELISA plate with 96 wells (Corning, USA) the iRBCs with 6% initial parasitemia were serially diluted by twofold with uninfected RBCs (previously washed with PBS) to obtain parasitemia range from 0.001 to 6%, ensuring a final volume of 200 µL of suspension. To each 200µL suspension 10µL of 20x SYBR Green I (diluted in PBS) working solution was added and was incubated for 30 min in the dark at 37ºC. Serially dilution was made in triplicate and in parallel uninfected culture (as a negative control) was turned in to 1-2,5 hematocrit, to compare the results.

Each serially diluted sample, including the negative control analyses were performed, in triplicate, in a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA) equipped with 488 nm (blue laser) and 638 nm (red laser) lasers, interfaced with a computer and the record data was analyzed using FlowJo platform v10.6.1. A gating strategy was used as a template to gate for the red cell zone of interest. Ten thousand events were recorded per sample. The number of gated events was recorded on a forward scatter (FSC) versus side scatter (SSC) dot plot and tracked in the FITC (fluorescein isothiocyanate) channel.

2.2. *Babesia ovis* protein isolation

Since *B. ovi*s is an intracellular parasite different protocols were performed in order to evaluate which method best results in terms of parasite isolation and protein purification. In parallel, extractions of protein from suspensions of 10% RBCs subjected to the same conditions of *B. ovis* culture, were performed to be used as control.

2.2.1. *Babesia ovis* isolation

2.2.1.1. Percoll gradient

The following methodology was adapted from the protocol described by Moeko et al. in “Application of Percoll density gradient centrifugation for separation of *Babesia ovata* infected erythrocytes” (Moeko, et al., 2017). Percoll gradient was achieved by preparing an aqueous solution of Percoll, that was diluted with Vega y Martinez solution (1x) to obtain solutions with densities ranging from 1.09 to 1.12 g/mL (four intermediate dilutions). One mL of each Percoll dilution was carefully poured to form layers into a 15 mL falcon tube. Finally, one mL of *B. ovis* culture (treated as described in 2.2.1.3.) was layered on top of the Percoll solution and then centrifuged at 2500 RPM

during 20 min at room temperature. After centrifugation, each relevant layer obtained (four) was collected and transferred to 1.5 ml sterile tubes. A smear of each layer was made and after rapid staining with Hemacolor®, each smear was examined under an optical microscope at 1000X amplification. Labeled tubes for each layer of Percoll were stored at – 20ºC for protein extraction procedure.

The *B. ovis* enriched layers from Percoll gradient were subjected to protein extraction. Using the previous separation method different protocols were used to proceed with the protein extraction which are described in the following sections. Hundred µL of the obtained *B. ovis* enriched first fraction was treated with saponin for RBC lysis as described in 2.2.1.2. and extracted with 1%SDS Tris-HCl buffer as described in 2.2.2.2. section and other 100 µL of this first layer were used for extraction with Tri-reagent as described in 2.2.2.3. section. From the second relevant *B. ovis* enriched fraction, 100 µL were treated with saponin lysis as described in 2.2.1.2. and extracted with 1%SDS Tris-HCl buffer as described in 2.2.2.3. section, 100 µL of third layer were subjected to Tri-reagent extraction method as described in 2.2.2.3. section and 100 µL of fourth layer were used for protein extraction with RIPA Buffer as described in 2.2.2.1. section.

2.2.1.2. Saponin Lysis to release parasites from infected RBCs

In order to disrupt the erythrocyte membrane to release the parasites, *B. ovis* culture was centrifuged 2500 RPM to obtain a pellet with the iRBCs, which was after resuspended in two vols of saponin 0.2% in PBS, vortex and incubated on ice for 10 min. To the resuspension, was added 1.5 mL 1x PBS and centrifuged at 4000 RPM. After removal of the supernatant, the addition of Saponin and PBS was repeated twice, under the same conditions described. The pellet was washed with 1.5 mL of PBS and centrifuged at 14000 RPM for 10 min. Supernatant was discarded and the pellet was stored at -80 º C until the protein extraction with 1%SDS Tris-HCl buffer, described in 2.2.2.3. section.

2.2.1.3. Red blood cells mechanical lysis followed by filtration

Approximately 1 mL of *B. ovis* culture (>6% parasitemia) was placed in 1.5 mL microcentrifuge tube and centrifuged for 5 min at 1500 RPM. Complete medium was

added in a 1:1 proportion to the pellet and aspirated into a sterile syringe with an 26G needle. Ten successive aspirations were performed, promoting RBC lysis and the release of parasites to the medium. The suspension was after centrifuged for 2 min at 4000 RPM, to retrieve *B. ovis* parasites in the supernatant. To remove RBC debris, the supernatant was afterwards filtered, using either a syringe membrane filter pore size 1.2 µm and 32 mm diameter (GE Whatman™) or a syringe membrane filter pore size 2.0 µm and 50 mm diameter (VWR). In the latter case medium was added to the isolate before filtration to increase volume and insure passage through the filter. Subsequently the protein was extracted with Ripa Buffer as described in 2.2.2.1. section.

2.2.1.4. CO_2 deprivation

B. ovis parasites cultured in 4 mL of culture medium containing 10% lamb RBC (RBC suspension) in 6-well plates (Corning Costar, Corning, New York, USA) were afterwards placed in a 37ºC chamber without CO_2 atmosphere, during 4 to 6 hours for spontaneous release of merozoites, according to the protocol previously described by Levy & Ristic, 1980 (Levy & Ristic, 1980). The total volume of culture that was deprived for CO_2, after 6 hours was decanted to a 50 mL falcon tube and centrifuged for 5 min at 3500 RPM. The supernatant was collected and centrifuged two times during 10 min at 2500 RPM and one last time during 20 min at 3000 RPM. Finally, the sediment was kept, resuspended with 199 Medium (Gibco, Thermo Fischer Scientific) and suspension was filtrated using a sterile syringe membrane filter with pore size 1.2 µm and 32 mm diameter (GE Whatman™) to obtain the free merozoites. The final sample was stored at -20ºC for further protein extraction with RIPA Buffer as described in 2.2.2.1. section. The presence of free merozoites was evaluated by the visualization of a Hemacolor® stained smear.

2.2.2. Protein extraction

As previously referred, different protocols for total protein extraction were performed, which are detailed in the following sections.

2.2.2.1. RIPA cell lysis buffer

Radioimmunoprecipitation assay buffer (RIPA buffer) is a widely used reagent for total cell lysis of adherent or nonadherent cells in culture and protein purification. Briefly, RIPA buffer contains a denaturing lysis nonionic detergent NP-40, plus two ionic detergents, sodium deoxycholate and SDS that promote cell lysis and enables the release of cell proteins. Herein, first *B. ovis* cultures were centrifuged at 17000 RPM for 15 min at 4ºC, the supernatant was discarded and to the pellet was added 5 µL of protease inhibitor cocktail M221 (VWR) and 50 µL of RIPA buffer (VWR, Amresco). The resuspended pellet was kept in ice for 15 min in slow agitation. The resuspension was centrifuged at 16000 RPM for 15 min, the supernatant was transferred to a previously cooled 1,5 mL tube and stored at -20ºC, until protein analysis.

2.2.2.2. Protein extraction with 1%SDS Tris-HCl buffer

The enriched *B. ovis* fractions, previously obtained (described in section 2.2.1.1), were resuspended in 100 µL of SDS lysis buffer with 10 µL of protease inhibitor cocktail M221 (VWR). After, the suspension was sonicated (J.P. Selecta, Barcelona), twice, on ice for 2 min. After sonication, the mixture was centrifuged at 14000 rpm during 30 min at 4ºC. Supernatant containing the total lysate proteins was collected and kept at -80ºC for further protein analysis.

2.2.2.3. Protein extraction with TRI-reagent

The enriched *B. ovis* fractions, previously obtained (described in section 2.2.1.1), were resuspended in 200 µL of TRI reagent (Sigma-Aldrich). Repeated pipetting was performed in order to promote cell lysis and after the manufacturer instructions were followed. Briefly, after removing the aqueous phase (for RNA isolation) 60 µL of 100% ethanol were added to the interphase and organic phase (containing DNA and proteins). After samples were mixed by inversion and centrifuged at 6000 RPM for 5 min at 4ºC. Supernatant was collected and protein precipitation was achieved by adding 300 µL of 2-propanol, followed for centrifugation at 15000 RPM for 10 min. The supernatant was discarded, and the pellet washed three times with 400 µL of 0.3 M guanidine hydrochloride/95% ethanol solution. Finally, the mixture was washed in 100% ethanol, centrifuged at 11500 RPM for 5 min at 4ºC. Pellet was dried for 15min at room

temperature and dissolved 1% SDS. The protein suspension was promptly used or was stored at -20ºC until further use.

2.3. Synthesis of a *Rhipicephalus bursa* protein: Propionyl-CoA carboxylase

A 37 a.a peptide (VKTPEECVKIAQSIGYPVMIKASAGGGGKGMRIAWND) based on a tick protein (UniProt ID: L7MAU7) was synthetically produced by GenScript Corporation (Piscataway, NJ, USA). To increase peptide immunogenicity, an Imject™ Blue Carrier™ Protein (highly soluble, mollusk-derived hemocyanin) (Pierce Biotechnology Inc., IL, USA) was conjugated with the peptide using one step glutaraldehyde conjugation (previously prepared under Joana Couto PhD studies).

2.4. Quantitative and qualitative evaluation of protein extracts

2.4.1. Quantification with Nanodrop

The concentration of protein extract was determined by spectrophotometry using the NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, USA). For this 2 µl of protein extract was placed in the apparatus and light absorption at 280 nm was measured.

2.4.2. Quantification with Qubit®

The concentration of protein was also evaluated using the Qubit® Fluorometer and the Qubit® Protein Assay Kit (Invitrogen, Thermo Fisher). The Qubit® Protein Assay Kit is designed to calculate the protein concentration within the Qubit fluorometer using a pre-programmed assay. The kits include the concentrated assay reagent, the dilution buffer, and the pre-diluted BSA standards. Manufacturer instructions were followed. Briefly, a work solution was prepared by diluting the concentrated assay reagent using the dilution buffer provided and after this solution was added to the samples and BSA standards. After an incubation of 15 min at room temperature, the samples were analyzed in the Qubit® Fluorometer.

2.4.3. SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis)

To evaluate the quality of the previously obtained protein extracts, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. This technique is widely used to separate complex mixtures of proteins, to investigate subunit compositions, to verify homogeneity of protein samples or even to purify proteins for usage in further applications. The standard Laemmli method is used for discontinuous gel electrophoresis under denaturing conditions, that is, in the presence of sodium dodecyl sulfate (SDS).

Protein sample Laemmli Loading buffer (VWR, USA) was added to the protein samples in a 1:6 proportion. These samples were incubated at 99ºC for 10 min to promote protein denaturation and loaded into the SDS-PAGE gel wells. SDS- PAGE gels were prepared according to the table 1 (12,5% discontinuous (5% stacking gel, 12,5% separating gel).

SDS-PAGE gels were stained with BlueSafe (NZYTech, Lisbon, Portugal) at least for 30 min and after washed with milliQ H_2O with rocking.

Table 1: Preparation of SDS-PAGE gel - 12,5% separating gel and 5% stacking gel

	12,5% Separating gel		**5% Stacking gel**
	For 1 gel	For 2 gels	
H_2O Milli Q	2,988 mL	5,975mL	2,825 mL
Acrylamide	3,125 mL	6,250 mL	0,625 mL
Tris-HCL 3M (pH=8,8)	0,938 mL	1,875 mL	---
Tris-HCL 0,5M (pH=6,8)	---	---	1,25 mL
10% SDS	75 µL	150 µL	50 µL
TEDED	3,75 µL	7,5 µL	3,75 µL
10% Ammonium persulphate solution (APS)	37,5 µL	7 5µL	25 µL

2.5. Production of polyclonal and monoclonal antibodies against a tick protein and *Babesia ovis* protein extract

2.5.1. Mouse immunization with *Babesia ovis* protein extract and PCCA peptide

For polyclonal/monoclonal antibody production CD1 male mice (reared and maintained at IHMT animal house) with 3-4 weeks (three mice per protein) were immunized intraperitoneally with 20 µg of protein extract or 20 µg of synthetic peptide – PCCA, every 2-3 weeks. Pre-immune serum was collected before immunization and was used as negative control to evaluate the antibody production. For immunizations protein extract and recombinant peptide were emulsified with incomplete Freund's adjuvant (Sigma-Aldrich, MO., USA) in a 1:1 proportion. Indirect enzyme-linked immunosorbent assay (ELISA), after described, was used to monitor the serum titers of each mouse after each immunization. For that, serum from mandibular vein blood of each mouse was obtained before each inoculation. The mouse with higher antibody titer was selected to proceed to cell fusion and, 3 days before euthanasia, it was subjected to a final boost. The mouse spleen was removed under sterile conditions, kept in DMEM (Dulbecco´d Modified Eagle Medium) and used promptly. Mouse total blood was also collected in order to obtain antiserum, which is extremely rich in antibodies against the protein extract or recombinant peptide, respectively.

2.5.1.1. Antibody titer monitoring of mouse serum using ELISA

Blood collected from mandibular vein of the mice was separated by centrifugation at 4500 RPM for 10 min, serum was collected to a sterile 1,5 mL tube and kept at -20 ºC until use. A high binding 96 wells ELISA plate (Corning® Costar®, MA, USA) was coated overnight at 4ºC with 0.1µg of protein extract or PCCA diluted in PBS (100µl per well). For the negative control, wells were incubated only with PBS. Subsequently plates were washed with Tris buffered saline (25 mM Tris HCl, 150 mM NaCl, and 2 mM KCl) containing 0.05% (v/v) Tween 20 (TBS-T), blocked with 300 µl of 5% (w/v) milk (Bio-Rad, Hercules, CA, USA) at room temperature for 90 min and washed five times with TBS-T). The first antibody, mice serum (diluted 1:200 in PBS), was incubated for one hour at 37ºC and wells were afterwards washed three times with TBS-T. The secondary antibody anti-Mouse Polyvalent Immunoglobulins alkaline phosphatase (AP) conjugated (Sigma-Aldrich, St. Louis, Missouri, USA) (diluted 1:5000 in TBS-T), was added to the wells, incubated for one hour at 37 ºC and wells were washed five washes with TBS-T. Washed plates were finally incubated with 1 mg/ml of p-nitrophenyl phosphate in substrate buffer (100 mM glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, and pH 10.4) at room temperature in the dark. Absorbance was measured

at a wavelength of 405 nm in an ELISA plate reader (Triad Series Multimode Detector, Dynex Technologies, Chantilly, VA, USA) with Concert-Triad Series software (version 2.1.0.17).

2.5.2. Generation of Hybridomas

2.5.2.1. Maintenance of myeloma cells

Cryopreserved cells were used to establish an adherent Sp2/0-Ag14 cell culture. Briefly, in the flow hood, cells were defrosted rapidly and resuspended in 40 ml of DMEM medium and centrifuged at 1200 RPM for 8 min. The pellet was then resuspended in 5 ml of complete medium (DMEM with 10% FBS (fetal bovine serum)) and transferred to a 25 cm^2 culture flask and incubated at 37 ºC and 5% CO_2 chamber. Cellular growth was monitored in an Olympus CK2 (Optical CO., LTD., Japan) inverted microscope. Every 72 h the medium was replaced by fresh medium, until a cell monolayer was visible. At this point the cells were transferred to 75cm^2 culture flasks and maintained until a new monolayer was reached, with cells in exponential grow. Cell culture was maintained in medium containing 8-azaguanine (Sigma-Aldrich) for 6-8 days to ensure its sensitivity to hypoxanthine-aminopterin-thymidine (HAT) A few days prior to fusion, azaguanine was removed from the medium by centrifugation and the cells were resuspended in fresh medium.

2.5.2.2. Cell fusion

Cell fusion was performed promptly after the spleen of the mice with higher titer had been aseptically removed under sterile conditions in a horizontal flow hood. The spleen cells were used to fuse with the myeloma cells in presence of polyethylene glycol (PEG) using classic Cell Fusion/Hybridoma production technique described next.

The spleen was washed 2-3 times in DMEM, in a petri dish, after, with the aid of 1 mL syringes coupled with 26 G needles, was pierced and injected repeatedly with medium in order to separate the cellular content to the suspension. The spleen cell suspension was then centrifuged for 10 min at 1200 RPM and cells resuspended in fresh DMEM. In parallel, SP2/0 cells were retrieved from a 75 cm^2 flask, centrifuged for 10 min at 1200 RPM and resuspended also in DMEM. Ten µL of resuspended cells (spleen and SP2/0) were used to determine cell density and viability with Trypan blue (0.1% in

PBS, Sigma-Aldrich) in an improved Neubauer hemocytometer counting chamber, using the formula that follows:

$$\text{Cell/mL} = \text{dilution factor} \times \text{number of cells in big square} \times 10^4$$

The cellular viability is obtained by calculating the percentage of cells that did not exclude the vital dye.

Cellular suspensions were mixed in a 1:10 proportion of SP2/0: spleen cells. The cellular mixture was centrifuged at 1200 RPM for 10 min and the supernatant discarded. To induce cellular fusion, 800 µl of PEG-DMSO (polyethylene glycol–dimethyl sulfoxide) was slowly added (1 min) and gently mixed with the cell pellet. After standing for about 1 min the PEG-DMSO was diluted: first by slowly adding and gently mixing 1 mL of DMEM and then by an adding 20 ml of DMEM over 5 min. Finally, the suspension was centrifuged at 1200 RPM for 10 min and the hybridoma cell pellet resuspended in 39 mL of DMEM medium supplemented with 10% FBS and HAT 1× (Sigma – Aldrich) which was distributed on 96-well cell culture plates, by placing 100 µL of the cell suspension in each well. Twenty-four hours after the cell fusion, 100 µL of 10% FBS and HAT 1× supplemented DMEM medium, was added to each well and plates were incubated at 37 °C in a 5% CO_2 incubator.

2.5.2.3. Hybridoma culture and screening

Hybridoma cells were allowed to develop in the 96-well plates in complete DMEM medium (10% FBS and HAT 1x) at 37ºC in a 5 % CO_2 incubator, changing the medium every two-three days. Approximately two weeks after cell fusion (or when large amounts of cells were visible), the supernatants of the cultures were used to screen against the protein extract or PCCA peptide by ELISA (section 2.5.1.1.) using the supernatant of each well as the first antibody in the ELISA assay. Wells that showed a positive result by ELISA were transferred to 24-well plates containing 1 ml of fresh complete medium, re-tested by ELISA and further tested by Western Blotting (section 2.6.1.). Confirmed positive cultures were further transferred to 25 cm^2 flasks in a total volume of 10 ml (described next). The screening for hybridomas in the assay with protein extract of *B. ovis* was done in parallel with immunofluorescence (section 2.6.).

2.5.2.4. Hybridoma expansion

One mL of complete medium (DMEM with 20% of FBS and 1x HAT), was previously added to wells of 24 well plates. Selected hybridomas were resuspended by pipetting up and down and transferred to the respective well in the 24 well plate. Every two-three days' hybridomas were monitored and if necessary complete medium (DMEM with 20% or 10% of FBS and 1x HAT) was replaced. About a week later the screening by ELISA was performed. Expansion to 25 cm^2 flasks was made using the procedure above described. If necessary hybridoma cultures could be transferred to 75 cm^2 culture flasks, in total volume of 40 mL using the same process (using a cell scraper to resuspend the adherent cells from the surface of the flasks).

2.5.2.5. Hybridomas storage

For cryopreservation hybridoma cultures were centrifuged at 1200 RPM for 10 min, the cell pellet was resuspended in 1ml of FBS with 4% DMSO and aliquoted in cryovials which was stored immediately at -80 °C for 24 hours before being transferred to liquid nitrogen tanks for long term storage. This process was performed when necessary *e.g.* to limit cell expansion. To reestablish frozen cultures, the process is the same as already described in 2.5.2.1. section.

2.5.2.6. Monoclonal antibodies production: cloning by limiting dilution

The hybridomas screened and considered positive were further cloned, in order to produce cell lines with higher specificity towards the desired antigen. The selected hybridomas were evaluated to their density and viability with Trypan blue (0.1% in PBS, Sigma-Aldrich) in an improved Neubauer hemocytometer counting chamber. The cell concentration was obtained and the hybridoma culture was diluted to obtain, in theory, 0.5 cell per well (plating a 200 µl aliquot/well), in a 96 culture cell plates, to ensure growth from a single cell and thus, the production of monoclonal antibodies. Cell growth was followed closely, to distinguish between wells with a high amount of hybridomas or wells with approximately one hybridoma cell. Supernatants were screened by immunofluorescence as described below in section 2.6.. If appropriate, cells were expanded, cultured and stored as described above.

2.6. Evaluation of antibodies: indirect immunofluorescence

B. ovis culture slides were used to screen for anti-*B.ovis* antibodies. Uninfected RBCs were used as negative control. The slides were fixed with the first solution of Hemacolor® - methanol. After, the cells were permeabilized with 0.5% (v/v) Triton X-100 in PBS for 30 min and washed three times with excess PBS before blocking overnight with 5% (w/v) BSA at 4ºC in a humidity chamber. After washing the slides again with excess PBS, the primary antibody (hybridoma supernatants) were applied and the slides incubated for 1 hour at 37ºC. The slides were then washed three times with PBS and the appropriate secondary antibody, Alexa Fluor 488 (green) - conjugated anti-mouse diluted (1:100) in blocking solution, was applied and slides incubated for 1 hour at 37ºC. After a final PBS wash, a drop of ProLong® Gold Antifade Reagent with 4′, 6′-diamidino-2-phenylindole (DAPI) (Invitrogen) was placed over the smear and then slides were covered with a coverslip and sealed with nail polish. Slides were kept in a moist dark box until microscopic analysis to prevent drying and loose of fluorescence. Slides were visualized under a Nikon eclipse 80i fluorescence microscope with appropriate filter (GFP and DAPI and photographed with a Nikon DS-Ri1 camera (Nikon Europe, Amsterdam, Netherlands) coupled to the microscope.

2.6.1. Western blot

Western blot (WB) is often used in research to separate and identify proteins.

For WB analysis, proteins were transferred from a SDS-PAGE gel to a nitrocellulose membrane (Trans-Blot® Transfer Medium pure cellulose membrane (0.45 μm), (Bio-Rad, Hercules, California, USA), using Mini Trans-Blot® Electrophoretic Transfer Cell (BioRad) with transfer buffer composed of (20% (v/v) methanol, 25 mM Tris, 192 mM glycine in H_2O) for 1 hour at 50 volts or overnight at 20 volts at 4ºC. Briefly, the transfer module was assembled by placing a layer of sponges, two layers of filter papers, the protein gel, covering it with the prepared membrane, two filter papers and another layer of sponges. The nitrocellulose membrane and all filter papers and sponges were pre-soaked in transfer buffer, prior to assembly. Assembled “cassette” was placed on the transfer apparatus and transfer buffer was added to cover the “cassette”.

Transfer efficiency was determined by staining the membrane with in Ponceau Red 0.2% (w/v) diluted in acetic acid 3% (v/v), before being washed with dH_20 to visualize the transferred proteins. Membranes were cut into strips and after blocked with 5% (w/v) powdered skimmed milk (Biorad) in PBS -T buffer for 1 or 2 hours at room temperature or overnight at 4ºC, shaking. Subsequently, the strips were washed 3 times with 2 ml of TBS-T for 15 min each wash. Strips were incubated in 1 ml of primary antibody, that was the supernatant of each selected hybridoma, for 1 hour or overnight at 4ºC shaking. After incubation with the primary antibody, membranes were washed three times for 5-10 min and incubated with 1ml of the secondary antibody, anti-mouse antibody solution HRP conjugated (1:2000 in PBS-T) (BioRad) for 1 hour or overnight at 4ºC. After three washes with TBS-T, labeled proteins were revealed by applying an ECL (enhanced chemiluminescent substrate) (GE Healthcare Amersham, UK). Chemiluminescence was visualized by exposing the membrane to chemiluminescence film (GE Healthcare Amersham hyperfilm ECL) during different time periods. Films were revealed manually by immersion of films in developer during 5 min with agitation (Agfa Healthcare, Belgium), followed by immersion in fixer (Agfa) during 5 min with agitation. Films were after washed in ultrapure water and allowed to dry at room temperature. Results were visualized under a white light and photographed using a standard camera.

Results

3.1. Isolation of *B. ovis* and protein extraction

As an intraerythrocytic parasite it is crucial to optimize the isolation of *B. ovis* in order to reduce/minimize the contamination with host cell proteins. Herein, different protocols for parasite isolation were performed followed by standard protein extractions so that the best fractions of *B. ovis* protein extracts could be used to proceed with mice immunizations. *B. ovis* cultures of approximately 6% parasitemia were used in all cases. Control samples, that comprise uninfected RBCs, were used to compare results. Table 2 shows the obtained quantities of protein according to the combinations of isolation and protein extraction methodologies.

Table 2: Fluorometric (Qubit®) and spectrophotometric (Nanodrop®) quantitation of proteins fractions obtained according to different protocols.

Extracts	Isolation methodology	Extraction method	Nanodrop (µg/µL)	Qubit (µg/µL)
Extract 1	Percoll Gradient (3th layer)/ Saponin Lysis	1%SDS Tris-HCl buffer	0.01	1.06
Extract 2	Percoll Gradient (4^{th} layer)/ Saponin Lysis	1%SDS Tris-HCl buffer	0.26	2.47
Extract 3*	Percoll Gradient (3th layer)	RIPA Buffer	0.32	2.49
Extract 4	Percoll Gradient (4^{th} layer)	TRI-reagent	-0.53	0.924
Extract 5	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	0.16	---
Extract 6	RBC lysis/filtration (2,0µm pore)	RIPA Buffer	0.13	---
Extract 7*	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	0.42	4.66
Extract 8*	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	0.39	>5
Extract 9*	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	0.75	4.58
Extract 10 (negative control)	CO_2 deprivation. RBC lysis/filtration (1,2µm pore)	RIPA Buffer	0.88	4.1
Extract 11	CO_2 deprivation. RBC lysis/filtration (1,2µm pore)	RIPA Buffer	0.82	>5
Extract 12 (negative control)	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	0.81	4.12
Extract 13	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	0.85	>5
Extract 14	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	0.70	4.26
Extract 15*	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	0.76	5

Extract 16 (negative control)	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	---	4.20
Extract 17 (negative control)	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	---	3.84
Extract 18 (negative control)	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	---	4.72
Extract 19	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	---	4.66
Extract 20	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	---	3.98
Extract 21	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	---	4.28
Extract 22	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	---	4.56
Extract 23	RBC lysis/filtration (1,2µm pore)	RIPA Buffer	---	3.64

* Protein extracts used in mice immunization

Regarding the PCCA, based on the uncharacterized protein, UniProt ID: L7MAU7, a 37 peptide was synthetically produced and after reconstituted in ultra-pure water. Fluorometric quantitation showed a peptide concentration of 4.1 µg/µL.

3.2. Protein analysis – SDS-PAGE

The different protein extracts of *B. ovis* obtained were evaluated by SDS-PAGE, as shown in figure 6 and 7. Different extracts were tested and compared, showing a similar protein expression between different extracts. Plus, this analysis also demonstrates that the extracts have complex composition, mixture of proteins with different molecular sizes. In parallel, extracts of *B. ovis* culture were compared with negative culture extracts, to infer about differences in protein expression. It was possible to verify some quantitative differences in comparative terms, between *B. ovis* culture and negative culture. However, an identical protein distribution between both can be observed, probably indicating that the obtained *B. ovis* protein extract always contained a significant quantity of protein matter belonging to the RBC's.

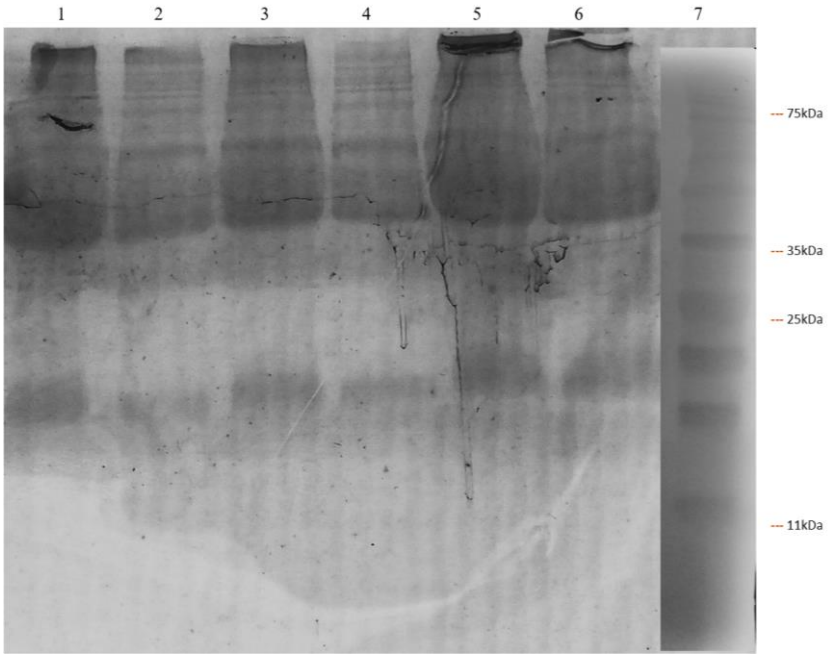

Figure 6: Electrophoretic separation of different protein extracts from *Babesia ovis* culture and negative culture.
Two µg of *B. ovis* protein extract and 2µg of protein extract of a negative culture were separated in 12% SDS-PAGE in denaturing conditions. Columns 1,3, 5 and 6: protein extract of *B. ovis* culture; 2 and 4: protein extract of negative culture. Column 7: NZYColour protein marker II.

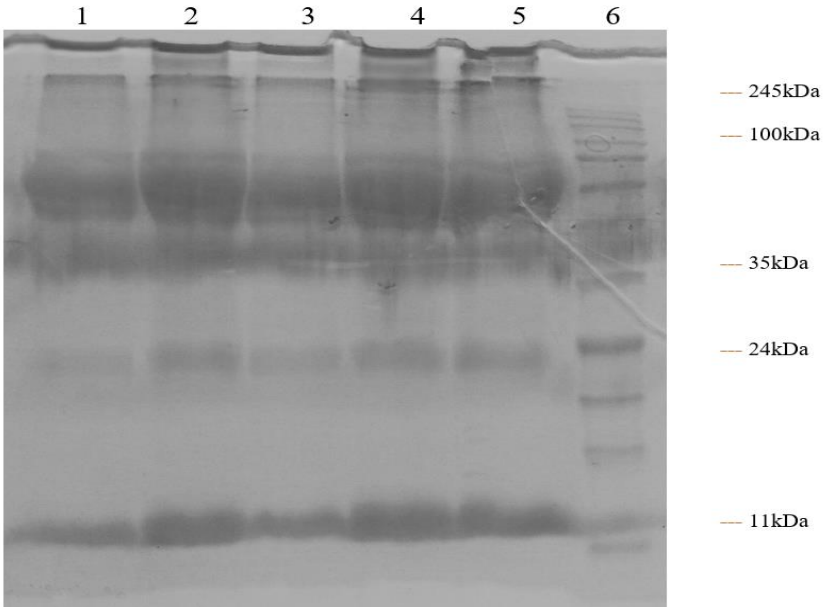

Figure 7: Electrophoretic separation of different protein extracts from *Babesia ovis* culture and negative culture.
Two µg of *B. ovis* protein extract and 2µg of protein extract of a negative culture were separated in 12% SDS-PAGE in denaturing conditions Colum 1: protein extract of negative culture as negative control; columns 2, 3 4 and 5: protein extract of *Babesia ovis* culture; column 6: NZYColour protein marker II.

SDS-PAGE analyses, for PCCA peptide, showed, as expected, a single band with low molecular weight, <11kDa (Figure 8).

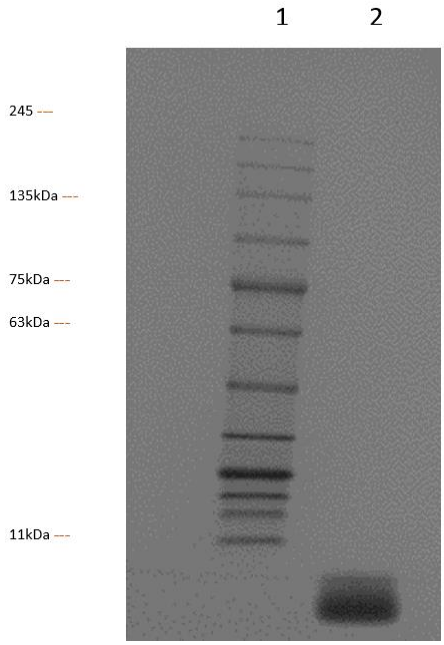

Figure 8: Polyacrylamide gel electrophoresis of PCCA.
Two µg of PCCA peptide was subjected to electrophoresis in 12% SDS-PAGE in denaturing condition. 1- Marker; 2 – PCCA peptide. (image provided by Joana Couto, PhD student)

3.3. Polyclonal and monoclonal antibodies production: mouse immunization with *Babesia ovis* protein extract and PCCA peptide

For generation of policlonal antibodies agaist *B. ovis* protein extract, three CD1 male mice 3-4 weeks old, were immunized intraperitoneally with 20 µg of protein extract, every 2-3 weeks. The extracts used for immunization are highlighted with * in table 2. Regarding the PCCA peptide assay, the same design and inoculation schedule was used.

During the immunizations process, blood samples were collected, and the serum titers against each of the inoculums were determined for each mouse by ELISA, using the *B. ovis* protein extract or recombinant peptide – PCCA as antigen. After four immunizations, ELISA results showed that the immune response against *B. ovis* protein extract and against the recombinant peptide – PCCA, were strong as showed in figures 9 and 10. In the case of immunization with the *B. ovis* protein extract assay, the serum of

each immunized mice namely R1, R2 and R3 were tested, as well, by immunofluorescence (Figure 11).

Results showed that the serum from mouse named R1 presented antibodies capable of recognizing *B. ovis*, thus it was selected for a final immunization (boost) and 5 days after was sacrificed and total blood and spleen were collected.

Concerning PCCA peptide, a work developed in parallel under Joana Couto PhD studies, had already used the two mice showing higher titers (designated R3 and R2), thus, R1 mouse was used in the present work, after a final boost immunization.

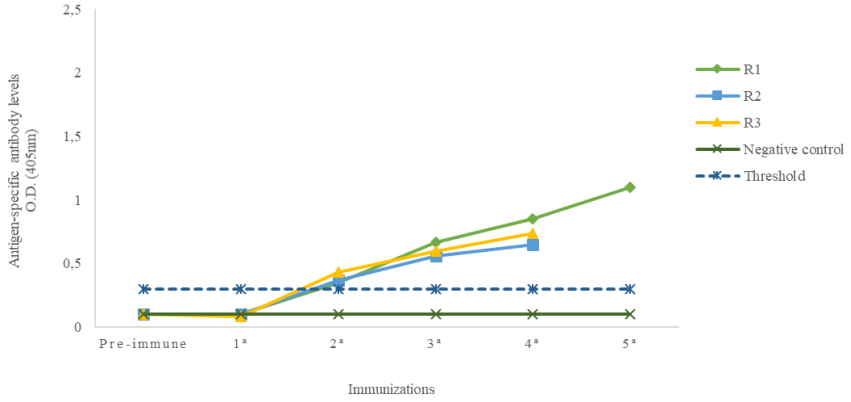

Figure 9: Determination of mice response to immunization with *Babesia ovis* protein extract by ELISA.
Three CD-1 mice R1, R2, R3 were inoculated with protein extract of *B. ovis* with incomplete Freud's adjuvant and blood collected prior to the inoculations. Mice serum was used to evaluate the immune response of each mouse by ELISA, in a dilution of 1:200. Pre-immunization serum was used as negative control. Threshold level represents the triple of negative control O.D. value.

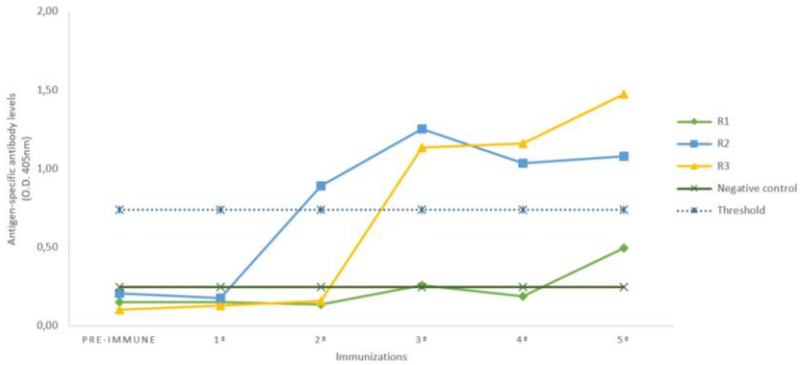

Figure 10: Evaluation of immune response against inoculums of recombinant peptide – PCCA.
Three CD-1 mice R1, R2, R3 were inoculated with peptide – PCCA with incomplete Freud's adjuvant, blood collected prior to the inoculations in selected mice for fusion. Mice serum was used to evaluate the immune response of each mouse by ELISA, in a dilution of 1:200. Pre-immunization serum was used as negative control. Threshold level represents the triple of negative control O.D. value (data provided by Joana Couto, PhD student).

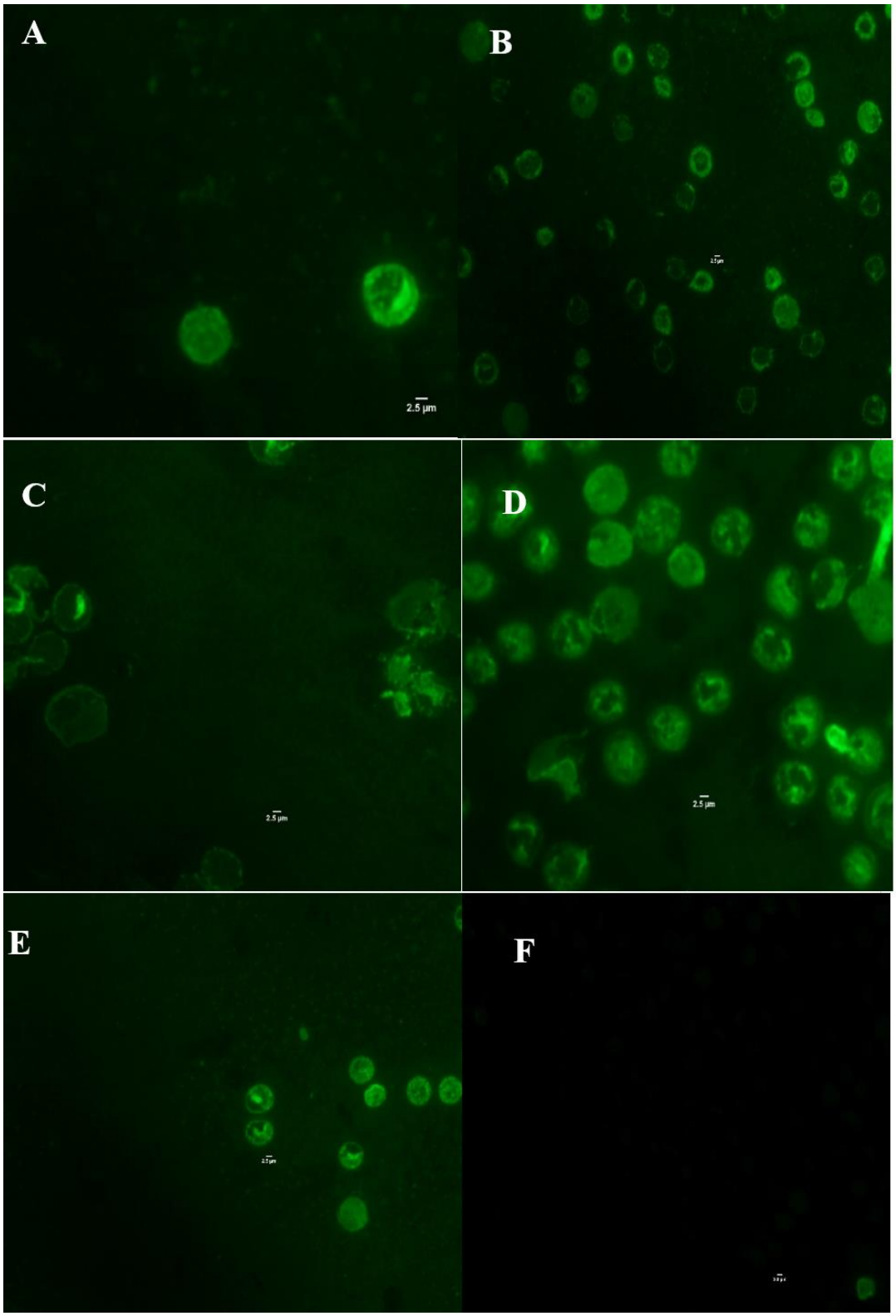

Figure 11: Immunofluorescence screening of anti-*Babesia ovis* serum.
Babesia ovis culture smears were incubated with serum (1:200) collected from immunized mice R1 (E, D), R2 (C) and R3 (A, B) and pre-immune serum (F). Anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody and slides visualized under a Nikon fluorescence microscope under a GFP filter at 100x amplification. Scale bars 2,5 µm.

Afterwards, two parallel cell fusions were performed to obtain hybridomas antibody producers, against *B. ovis* protein extract and hybridomas antibodies producers against the recombinant peptide - PCCA. Both cell fusions were successful with hybridomas starting to appear a few days after cell fusion. Approximately 14 days after cell fusion supernatants from the 96 well plates were used to screen for antibody production against the target extract or peptide by ELISA, resulting in 70 hybridomas growing in the 384 wells seeded in the *B. ovis* protein extract case and 41 wells with hybridomas growing among the 384 seeded in the PCCA peptide fusion. The positive wells were selected for expansion, initially to 24 well plates and subsequently to 25cm^3 culture flasks, after new screening by ELISA (Table 3).

Table 3: Results from supernatant screening.
Babesia ovis protein extract or PCCA peptide were used to cover a high-binding ELISA plate, and supernatants from hybridoma cultures were used as primary antibodies. Supernatants from hybridoma cultures were considered positive when showing O.D. values above triple of the negative control.

	96 wells plates		**24 wells plates**		**25cm^3 flasks**	
	Tested	**Positive**	**Tested**	**Positive**	**Tested**	**Positive**
Babesia ovis protein extract	384	70	48	8	8	4
PCCA peptide	384	41	40	5	5	3

Once in a 24 wells plate, *B.ovis* protein extract hybridomas were left to grow approximately 7 days and after supernatants were tested by ELISA and the positive ones were tested by immunofluorescence assay and by Western blot to assess their specificity to *B. ovis* proteins (Table 4).

The same approach was used with the PCCA peptide and three hybridomas were selected **PCCA-4A12**, **PCCA-3B12** and **PCCA-3D12** for expansion to 25 cm^3 flasks and tested by Western blot to assess their specificity.

Table 4: Hybridoma screened by IFA.
Supernatants from hybridoma culture tested by IFA presenting fluorescence, marking *Babesia ovis*.

	24 wells plates		**25 cm^3 flasks**	
	Tested	**Positive**	**Tested**	**Positive**
***Babesia ovis* protein extract**	8	3	3	3

Eight *B. ovis* protein extract hybridomas **(BOPE),** selected by ELISA, were evaluated by immunofluorescence. Positive supernatants from each assay were stored at -20ºC for further use and hybridomas were stored at -70ºC.

3.4. Immunofluorescence assay

This assay allowed to infer about the capacity of produced antibodies to recognize *B. ovis* proteins and/or RBC's proteins. A negative culture smear was used in parallel to validate results and to determine auto-fluorescence. According to the results, **BOPE 1A3**, **BOPE 2H5** and **BOPE 4F12** hybridomas were selected from a total of 8 tested hybridomas (Figure 12 to 20). These hybridomas were used to proceed to monoclonal antibodies generation.

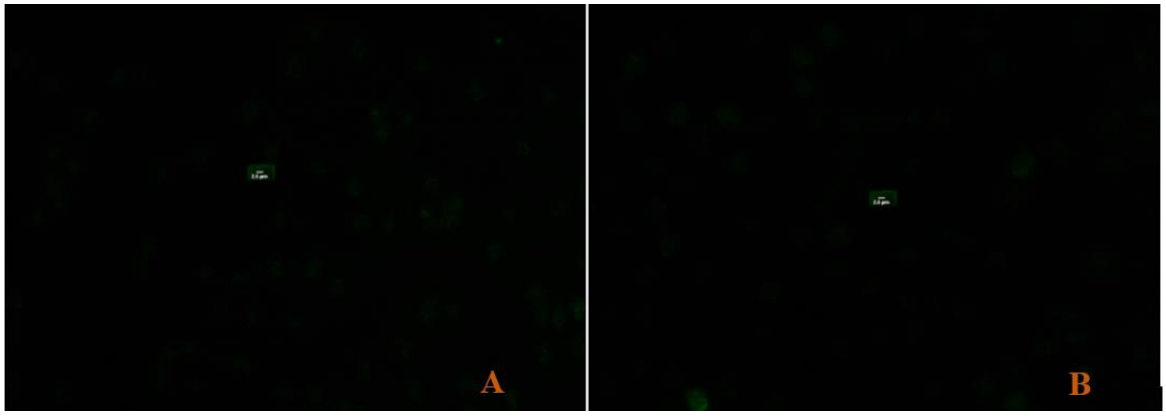

Figure 12: Immunofluorescence analysis in *Babesia ovis* culture smears – A and negative culture smears – B.
Pre-immune serum was used as primary antibody and anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody. Visualization was performed under a Nikon fluorescence microscope using a GFP filter at 100x amplification. Scale bars 2,5 µm

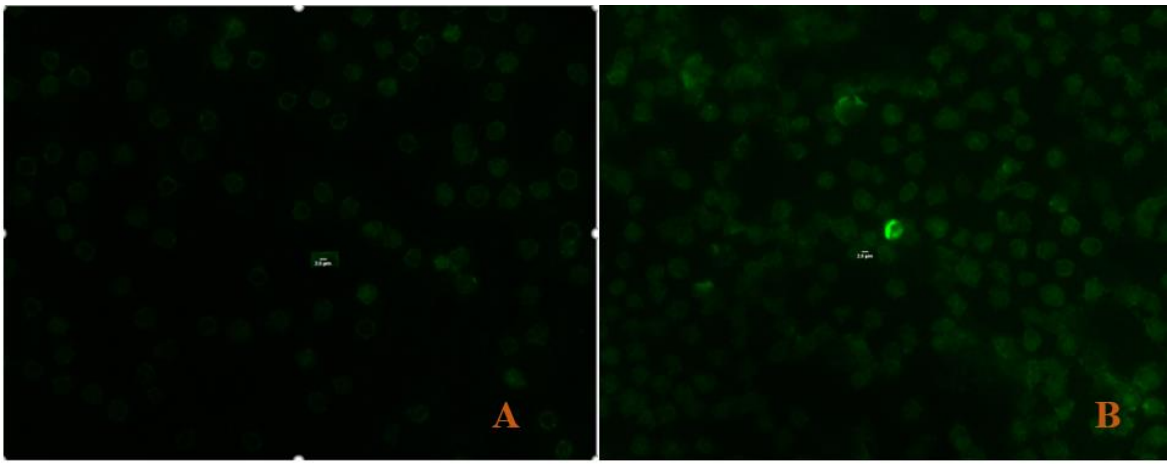

Figure 13: Immunofluorescence analysis in negative culture smears – A and *Babesia ovis* culture smears – B probed with produced antibodies by hybridoma BOPE 1A3.
Smears were incubated with supernatant of BOPE 1A3 hybridomas as primary antibody and anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody. Visualization was performed under a Nikon fluorescence microscope using a GFP filter at 100x amplification. Scale bars 2,5 μm

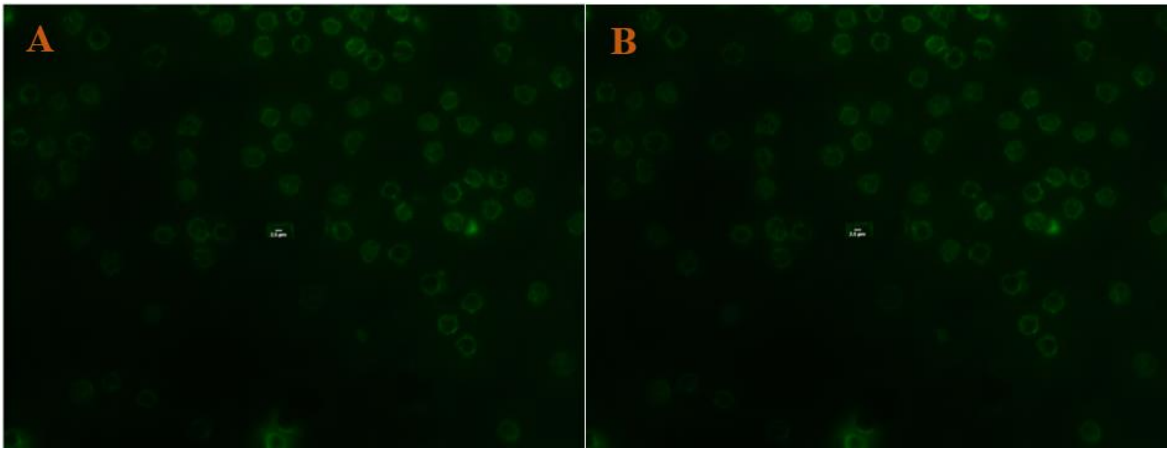

Figure 14: Immunofluorescence analysis in negative culture smears- A and *Babesia ovis* culture smears – B, probed with produced antibodies by hybridoma BOPE 1A4.
Smears were incubated with supernatant of BOPE 1A4 hybridomas as primary antibody and anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody. Visualization was performed using a Nikon fluorescence microscope under a GFP filter at 100x amplification. Scale bars 2,5 μm

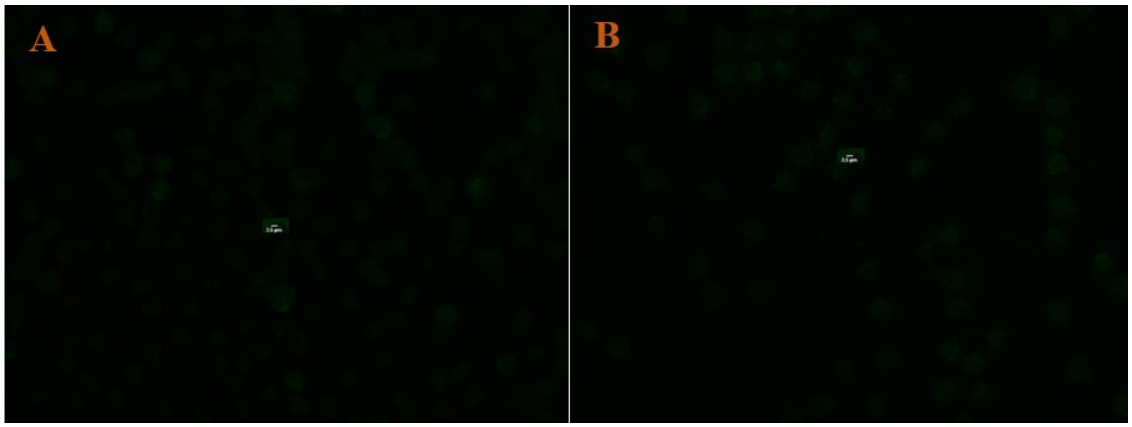

Figure 15: Immunofluorescence analysis in negative culture smears – A and *Babesia ovis* culture smears - B, probed with produced antibodies by hybridoma BOPE 1A6.
Smears were incubated with supernatant of BOPE 1A6 hybridomas as primary antibody and anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody. Visualization was performed under a Nikon fluorescence microscope using a GFP filter at 100x amplification. Bars 2,5 µm

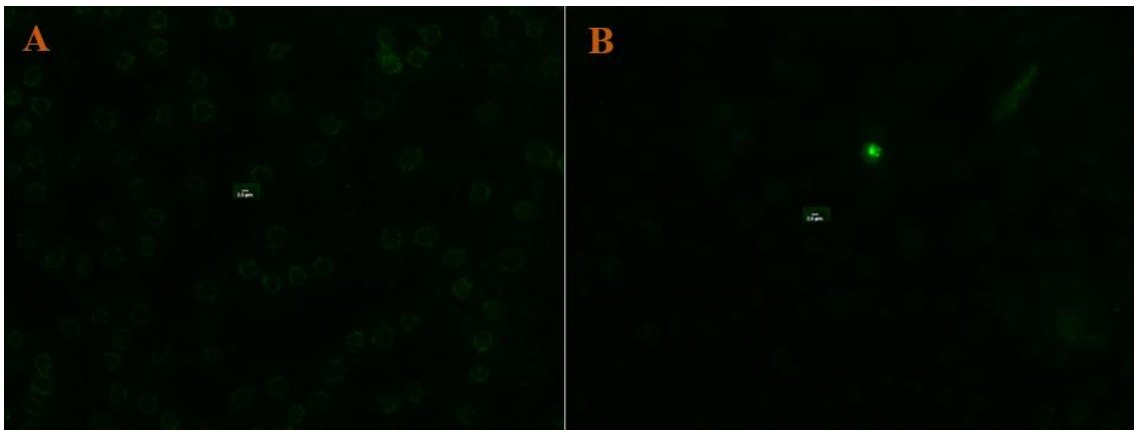

Figure 16: Immunofluorescence analysis in negative culture smears – A and *Babesia ovis* culture smears - B, probed with produced antibodies by hybridoma BOPE 2H5.
Smears were incubated with supernatant of BOPE 2H5 hybridomas as primary antibody and anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody. Visualization was performed under a Nikon fluorescence microscope using a GFP filter at 100x amplification. Scale bars 2,5 µm

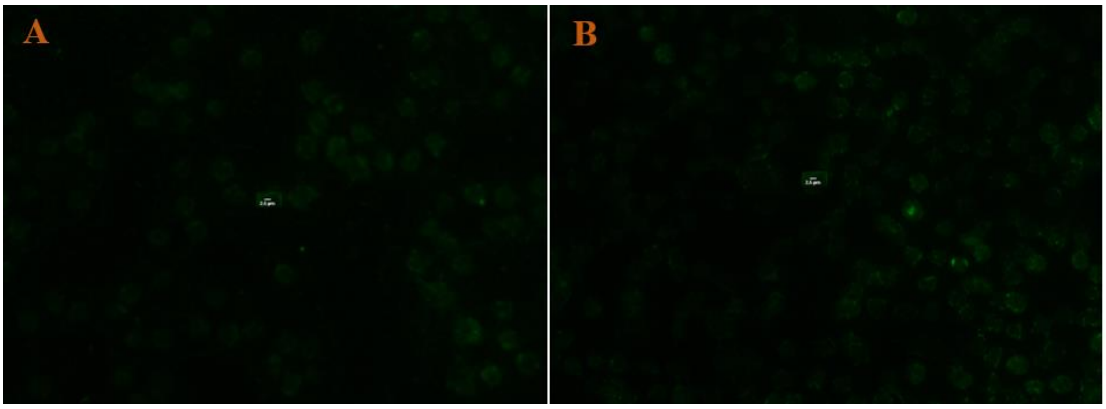

Figure 17: Immunofluorescence analysis in negative culture smears – A and *Babesia ovis* culture smears - B, probed with produced antibodies by hybridoma BOPE 4A11.
Smears were incubated with supernatant of BOPE 4A11 hybridomas as primary antibody and anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody. Visualization was performed under a Nikon fluorescence microscope using a GFP filter at 100x amplification. Scale bars 2,5 µm

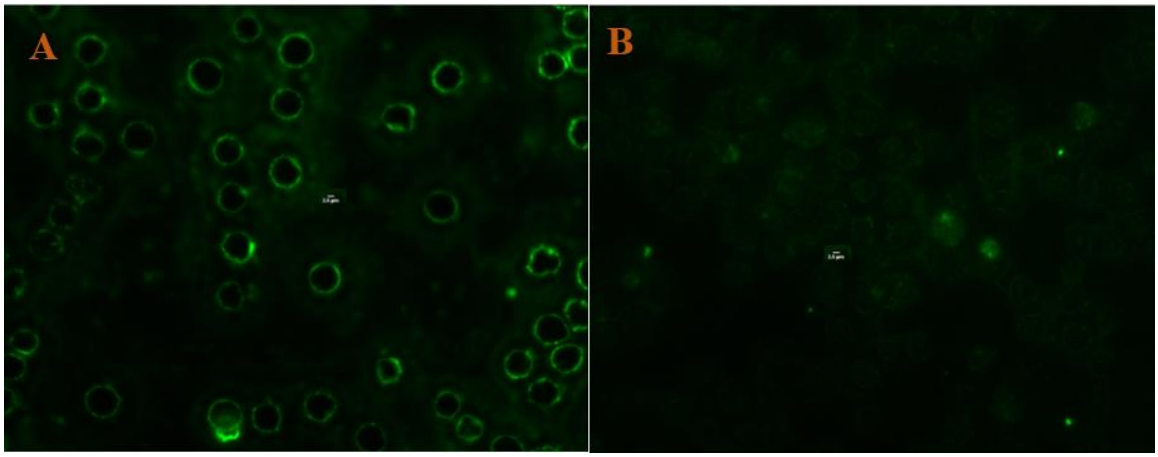

Figure 18: Immunofluorescence analysis in negative culture smears – A and *Babesia ovis* culture smears - B, probed with produced antibodies by hybridoma BOPE 4F12.
Smears were incubated with supernatant of BOPE 4F12 hybridomas as primary antibody and anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody. Visualization was performed under a Nikon fluorescence microscope using a GFP filter at 100x amplification. Scale bars 2,5 µm

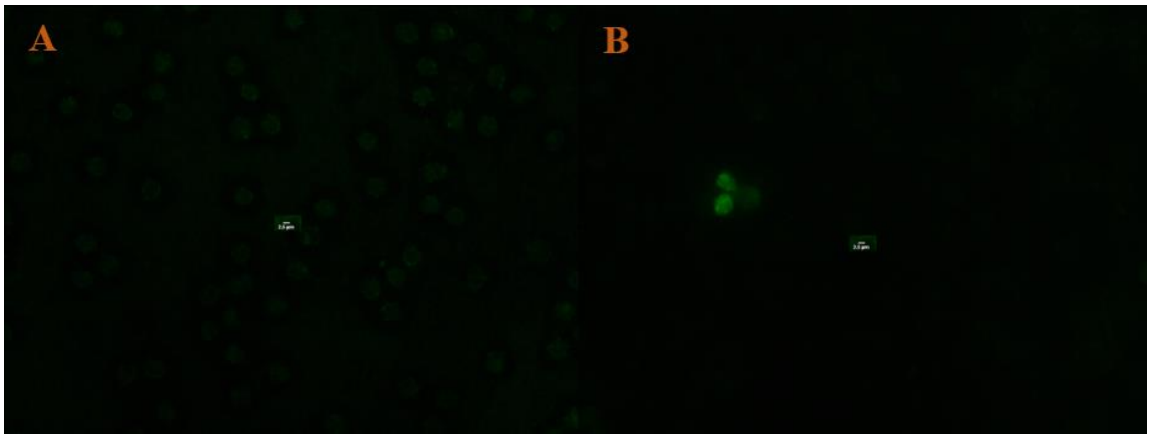

Figure 19: Immunofluorescence analysis in negative culture smears – A and *Babesia ovis* culture smears - B, probed with produced antibodies by hybridoma BOPE 4H12.
Smears were incubated with supernatant of BOPE 4H12 hybridomas as primary antibody and anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody. Visualization was performed under a Nikon fluorescence microscope using a GFP filter at 100x amplification. Scale bars 2,5 µm

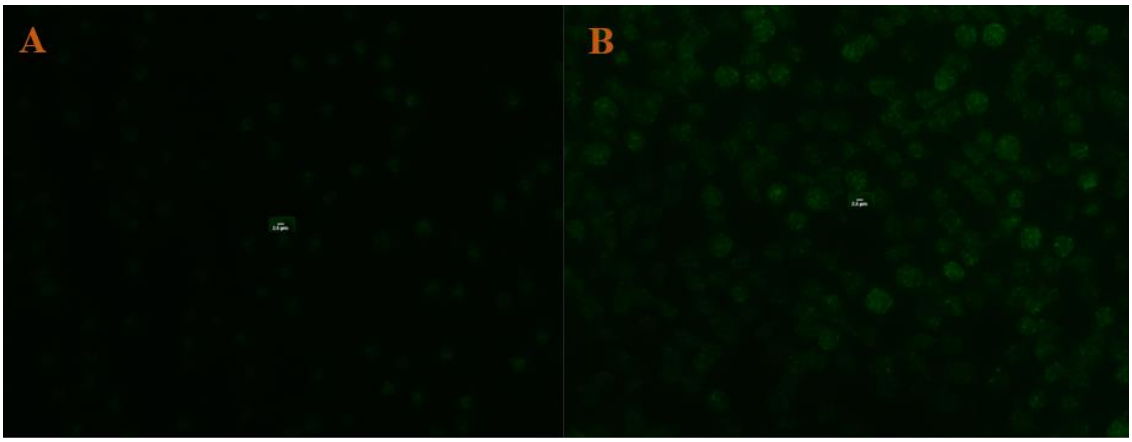

Figure 20: Immunofluorescence analysis in negative culture smears – A and *Babesia ovis* culture smears - B, probed with produced antibodies by hybridoma BOPE 4H5.
Smears were incubated with supernatant of BOPE 4H5 hybridomas as primary antibody and anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody. Visualization was performed under a Nikon fluorescence microscope using a GFP filter at 100x amplification. Scale bars 2,5 µm

3.5. Western-blot analysis

Polyclonal antibodies anti-*B. ovis* culture protein extract and anti-PCCA peptide were screened by WB to determine the recognition of proteins of interest. Recognition, of specific *B. ovis* proteins, was evaluated by comparing between a *B. ovis* culture protein extract and a negative culture extract, inferring about cross-reaction with RBCs proteins. Three hybridomas supernatants were tested, namely BOPE 1A3, BOPE 4F12 and BOPE 2H5, as shown in figure 21.

Supernatant of hybridoma BOPE1 A3 was probed against *B. ovis* protein extract and negative culture protein extract and a band of approximately 35 kDa was recognized in *B. ovis* protein extracts and was not in a negative culture extract, as shown in figure 22.

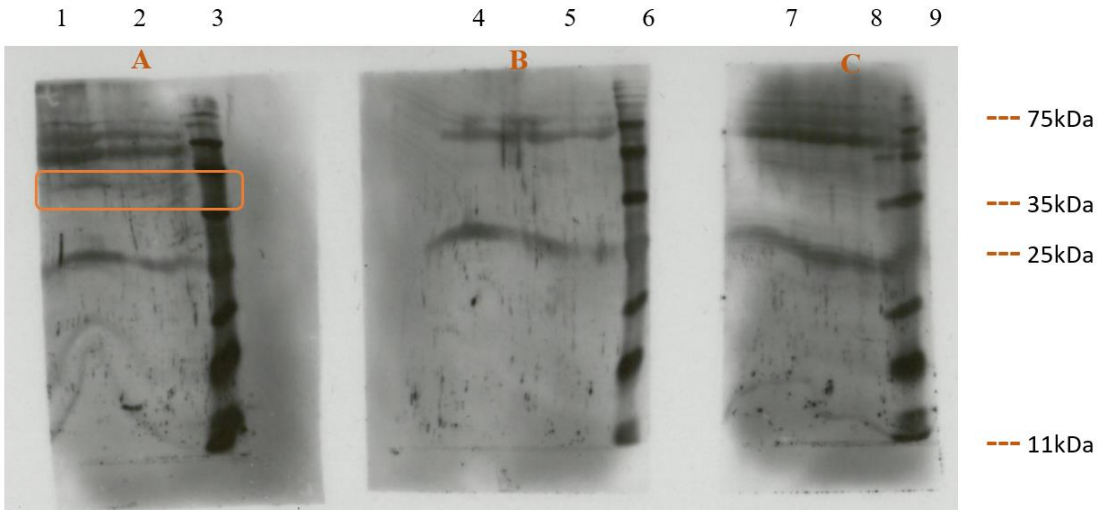

Figure 21: Screening of supernatants from hybridoma cultures by WB against *Babesia ovis* extract and negative protein extract.
Twenty µg of *B. ovis* protein extracts were subjected to electrophoresis, on a 12,5 % SDS-PAGE gels and transferred to a nitrocellulose membrane for WB with hybridoma supernatants as primary antibody and anti-mouse antibody solution HRP conjugated as a secondary antibody. ECL exposure time of 20 minutes Panel A- BOPE 1A3 hybridoma; Panel B- BOPE 2H5; Panel C- BOPE 4F12. Lanes 1, 4 and 7- *B. ovis* protein extract as antigen; 2, 5 and 8 - negative culture protein extract; 3, 6 and 9 – NZYColour protein marker II.

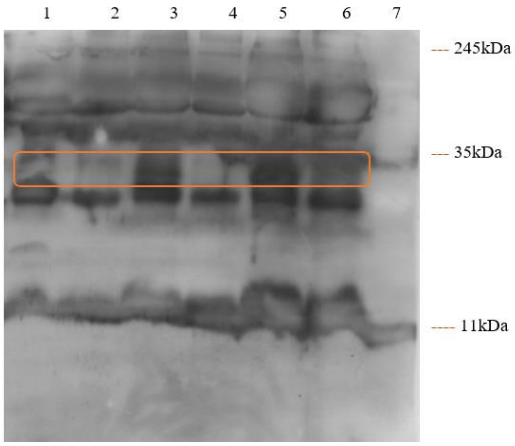

Figure 22: Screening of anti-BOPE 1A3 supernatant against different *Babesia ovis* protein extracts by WB.
Twenty µg of protein extract obtained from *Babesia ovis* culture were subjected to electrophoresis, on a 12, 5% SDS-PAGE gel and transferred to a nitrocellulose membrane for WB. BOPE1 A3 hybridoma supernatant was used as primary antibody and anti-mouse antibody solution HRP conjugated as a secondary antibody. ECL exposure time of 15 seconds. Lanes 1, 3, 5 and 6 correspond to *Babesia ovis* protein extracts. Lanes 2 and 4 are negative culture protein extracts. Lane 7 corresponds to the NZYColour protein marker II.

The recognition of PCCA peptide was also screened by WB. Three hybridomas supernatants were evaluated, namely PCCA 4D12, PCCA 3B12 and PCCA 4A12 as shown in figure 23. Results show that only supernatant PCCA 4D12 recognized the PCCA peptide, labeling a band of <11kDa.

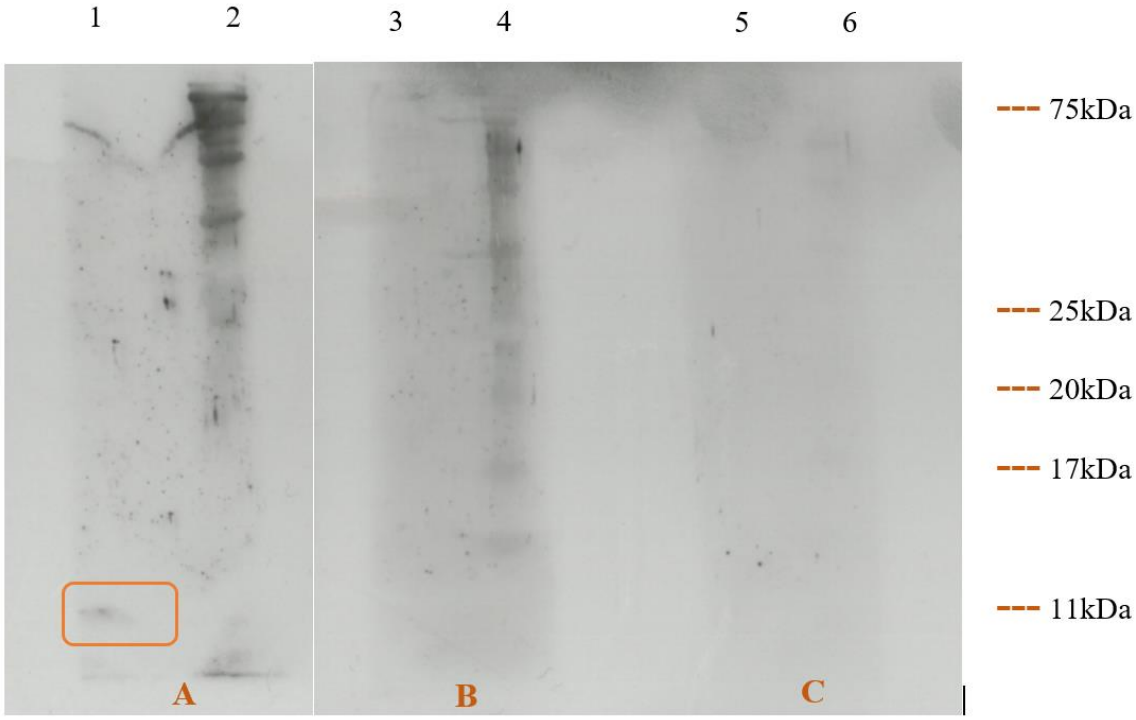

Figure 23: Screening of supernatants from hybridoma cultures by WB against PCCA peptide. Twenty µg of PCCA peptide were subjected to electrophoresis, on a 12,5 % SDS-PAGE gel, and transferred to a nitrocellulose membrane for WB. Hybridoma supernatants were used as primary antibody and anti-mouse antibody solution HRP conjugated as a secondary antibody. ECL exposure time of 15 minutes. Panel A corresponds to PCCA 4D12, B to PCCA 3B12 and C to PCCA 4A12. Lanes 1, 3 and 5 correspond to PCCA peptide as antigen and lanes 2, 4 and 6 to NZYColour protein marker II.

3.6.Monoclonal antibodies

After evaluation of specific antibody production, supported by ELISA screening, IFA and WB, the antibody-producing hybridomas, PCCA 4D12 and BOPE1A3 clones, previously frozen, were re-cultured. Cellular density and viability were evaluated, and it was observed that the cells were not responding well to the thawing process even after several attempts of recovery by changing medium, flasks and plates. Even though, it was decided to proceed with the wells in better conditions. The hybridoma cultures were diluted to obtain, in theory, 0.5 cell per well in a 96 culture cell plates, which allows cell cloning from a single cell and thus the production of monoclonal antibodies.

Cell growth was unsuccessful, and, in both cases, monoclonal antibodies could not be obtained even after repeating the entire process.

3.7.Flow cytometry

B. ovis culture parasitemia was determined by Flow cytometry assay, stained with SYBR Green I. A negative culture was used as control to show differences in population representation in infected and uninfected erythrocytes (Figure 24).

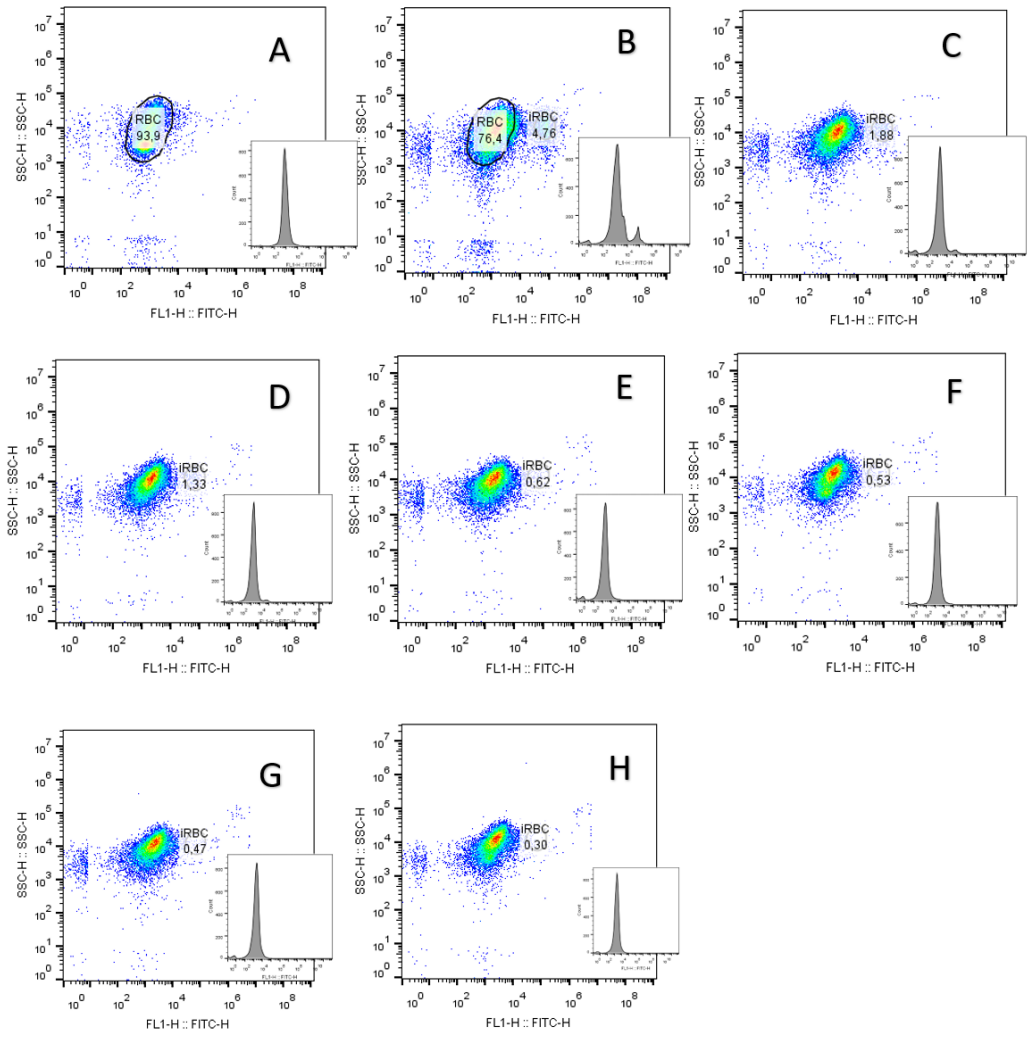

Figure 24: Parasitemia curve determination with flow cytometry with SYBR Green I staining, tracked in FITC channel.
A – negative culture as a negative control. **B** – *B. ovis* culture with 4% parasitemia**. C** to **H** – two-fold serial dilutions to obtain respectively 2%, 1%, 0,5%, 0,25%, 0,125%, 0,06% parasitemia. Histogram panel in B indicates the gated populations, that as either uninfected (RBC) and infected erythrocytes (iRBC).

A correlation, between FACS and Giemsa stained microscopic determinations of parasitemia with artificially diluted samples, was determined (Figure 25).

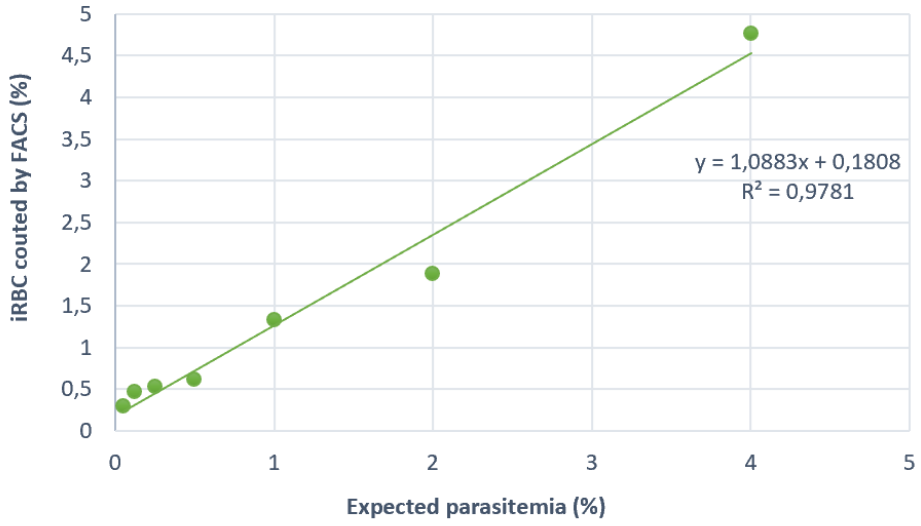

Figure 25: Correlation between FACS and Giemsa stained microscopic determinations of parasitemia with artificially diluted samples.
The line represents the linear regression whose statistical parameters were shown. Each point represented FACS measurement of iRBC and its corresponding microscopic manual count. Plotted parasitaemia ranged from 0.06% to 4.0% (expected values).

Discussion

The primary aim of the present study was to produce polyclonal and monoclonal antibodies against *B. ovis.* To achieve this, there was a need to optimize a protocol for isolation and extraction of protein from the parasite that would allow immunization with the cleanest possible inoculum, free of products related to RBC's present in the culture. Also, it was important to optimize a relatively easy and fast application protocol, because throughout the work it would be necessary to repeat the technique to obtain extracts not only for immunization but also for evaluation assays, such as ELISA or WB.

4.1. Protein – Isolation, Extraction and Quantification

Regarding the isolation process, the great difficulty resided on obtaining a fraction free of products belonging to the RBC's. *B. ovis* is an intraerythrocytic parasite and the presence of RBC debris is almost inevitable. In this way, different isolation techniques, previously described, were tested. Results have showed that Percoll gradient is a relatively limiting technique regarding the amount of sample in the layer of interest that contained free merozoites. Another technique of isolation applied was the deprivation of environment with CO_2, described previously, that suggested the spontaneous release of merozoites when a culture was subject to CO_2 deprivation (Levy & Ristic, 1980). This technique required the incubation in a chamber free of CO_2 for 4 to 6 hours. In addition to be a time-consuming process, after comparative analysis in blood smears of cultures submitted to this technique and cultures in a normal environment with 5% of CO_2, it was not possible register any major difference in the presence of free merozoites. It was also tested saponin lysis to release parasites from infected RBCs, which presented negative results in quantification with Nanodrop. The best technique for the release of the parasite was found to be the mechanical lysis, with the passage of culture (with parasitemia >6%) through a 26G syringe, which promotes the rupture of erythrocyte cell wall and consequent release of parasites to the medium. To clear the *B. ovis* fraction, a syringe filter of 1.2 µm was used since it was found. A greater pore filter, with 2.0 µm, resulted in the retention of the entire fraction on the filter, being necessary to add 199 Medium to recover a small part of the sample but losing most of the parasites in the process.

Protein extraction with RIPA buffer was found to be the most effective technique for protein extraction. This common buffer enables the extraction of

cytoplasmatic, membrane and nuclear proteins and is compatible with many applications, including, protein assays, immunoassays and protein purification. RIPA buffer contains a denaturing lysis nonionic detergent NP-40, plus two ionic detergents sodium deoxycholate and SDS. The combination of mechanical lysis followed by RIPA buffer extraction resulted in the best quantitation results. Regarding protein concentration evaluation, two quantitation methods were used: fluorometric and spectrophotometric. There was a need to find another quantification solution because the quantification with Nanodrop was being poorly reproducible. Although nanodrop quantification sometimes presents near-zero results, it was verified by the SDS-PAGE analysis that a greater amount of protein should be present. This possibility was tested with Qubit which uses a fluorometric quantification method (dyes binds specifically to protein). The quantification with Qubit presented more satisfactory and reproducible results.

To validate procedures and analyze the protein profile of different *B. ovis* culture extracts obtained, SDS-PAGE was used. Comparison of results shows a very identical protein expression in both the extracts, infected and uninfected RBCs indicating that all the purification methods cannot clear the RBCs debris.

In polyacrylamide gel electrophoresis, proteins migrate in response to an electrical field through pores in the gel matrix. The combination of gel pore size (pore size decreases with higher acrylamide concentrations) and protein charge, size, and shape determine the migration rate of the protein. The standard Laemmli method is used for discontinuous gel electrophoresis under denaturing conditions, that is, in the presence SDS (Gallagher, 2006). In the present study gel staining was performed using a Coomassie based reagent, the BlueSafe, which presents the safety advantage of not containing any methanol or acetic acid is a single step protein stain and like the Coomassie blue staining is very sensitive, acting in acidic conditions in the same way binding to basic and hydrophobic residues of proteins changing color from a dull reddish-brown to intense blue. As with all staining methods, staining detects some proteins better than others, based on the chemistry of action and differences in protein composition (Congdon, Muth, & Splittgerber, 1993). Thus, as little as 8–10 ng per band for some proteins and 25 ng per band for most proteins can be detected using the above described method. Such concepts may explain the similarity of results obtained while comparing protein extracts since RBC proteins presence can mask the *B. ovis* specific

proteins. Moreover, since protein extracts were used, proteins with proximal size but with different origins can appear in the same band.

4.2. Mice Immunization

In immunization, the quality of the protein extract to be used is of great importance, since the extract determines the specificity of immune response towards the proteins of interest. A Freud's incomplete adjuvant was used to enhance the immune response to the inoculum. The immune response to the inoculum was evaluated throughout immunizations, collecting blood before each immunization and later evaluation by ELISA. *B. ovis* protein extract immunization showed a weak positive result 6 weeks after the first immunization. At the end of the fourth immunization, ELISA results, figure 9, supported the selection of the R1 to procced for cell fusion since it presented a high titer against the inoculum. The selected mouse was subjected to a final immunization boost, immediately (3-4 days) before the fusion, to enhance the primary immune response.

Concerning the PCCA peptide, work was carried out in accordance with the work done by Joana Couto, immunization scheme showed in figure 10. As referred the R1 mouse was used in the present study, even though with weak immunization results since the remaining mice were not available.

4.3. Generation of polyclonal antibodies

To generate hybridomas capable of producing the desired antibodies, SP2/0 cells were fused to spleen cells of mice previously immunized with proteins of interest. For this, frozen stored SP2/0 cells were re-culture. However, cells took a long time to respond and to form a monolayer favorable to the realization of cell fusion. Two cell fusions were performed in parallel: the first using spleen cells of a R1 immunized mouse with *B. ovis* protein extract, and the other fusion was performed with spleen cells of R1 immunized mouse with PCCA peptide. After the two fusions, cells were maintained in 96 wells plates (four plates to each fusion) and cultures were analyzed after 2 weeks, by ELISA, to infer about the production of antibodies against *B. ovis* protein extract or PCCA peptide. The first screening by ELISA, according to table 3, allowed the selection of 70 polyclonal anti- *B. ovis* protein extract antibodies producer

hybrids from 384, and the 48 with higher absorbance values were expanded to 24 wells plates and screened again, two weeks after expansion, again by ELISA.

Based on the evaluation by WB, of the *B. ovis* protein extracts, a mixture of hybridomas producing antibodies against RBC proteins or *B. ovis* proteins was expected. Thus, immunofluorescence was used to select first the mouse serum that marked *B. ovis* proteins only/also (Figure 11) and after to compare supernatants from ELISA positive hybridomas. In that way, IFA assays allowed the selection of BOPE 1A3, BOPE 2H5 and BOPE 4F12 hybridomas from a total of 8 tested hybridomas. In the later cases, it was possible to observe that the supernatants from these 3 clones, along with RBC recognition also marked the parasite, apparently recognizing membrane proteins while the remaining did not clearly marked the parasite (Figure 13 to 20).

Due to time constrains, selected hybridomas and their respective supernatants were stored. Hybridomas were cryopreserved when showing exponential growth, favorable to the storage process and evaluation tests continued to select hybridomas with *B. ovis* specific recognition to process to production of monoclonal antibodies. Cryopreserved hybridomas did not respond well to re-culturing and their viability was never satisfactory. Several attempts were made to recover the hybridomas, including boost with the amount of serum used, preparation of fresh culture medium and use of commercial culture medium.

The hybridoma technology was validated performing the same technique for the PCCA peptide. Being a single peptide, free of another nontarget proteins, generation of hybridomas is more straightforward and, as a result, secreted antibodies successfully recognized the target peptide.

As mentioned, WB is often used in research to separate and identify proteins. In this technique a mixture of proteins is separated based on molecular weight, through gel electrophoresis. These proteins are transferred to blotting membrane, usually made of nitrocellulose or polyvinylidene fluoride, which binds to proteins. Polyacrylamide gel is placed over the membrane and the application of electrical current forces proteins to move from gel to membrane, where they adhere and can be subsequently linked to a specific antibody. The unbound antibodies are washed off, leaving only the bound antibodies to the protein of interest which can be subsequently detected by a chemical reaction that involves the release of energy in the form of light. The emitted light can be captured on an X-Ray film. As the antibodies only bind to the protein of interest, only one band should be visible. The thickness of the band corresponds to the amount of

protein present; thus, using a standard curve the amount of protein present can be estimated (Mahmood & Yang, 2012). In the present study, WB analysis demonstrated that the polyclonal antibodies recognized, among others, but differentially in relation to a negative extract, a 35kDa antigen in the *B. ovis* protein extract. It would be important to characterize the protein portion that is recognized and infer about its origin, characterizing it for the specific location of antigen linkage. Also, the effect of pAbs could also be evaluated in parasite growth, shedding light on the possible effect of blockade of such protein in parasite invasion and survival within erythrocytes.

4.4 Monoclonal antibodies

During the execution of the present study the production of mAbs failed. Possibly a novel cell fusion without the process of cell storage, in order to minimize the loss of antibody-producing cells, could be a solution. Another possibility could be the enrichment of the protein extract with *B. ovis* proteins since a "cleaner" extract, will allow a simpler and faster hybridoma selection. This pure extract could afterwards be submitted to SDS in order to separate by size proteins that could after being used for direct immunize mice. Initially the present study was thought to target isolated *B. ovis* membrane proteins which could had, in part, surpass some constrains, however the absence of an ultracentrifuge necessary to isolate such fraction of proteins precluded this option.

Although there are still few existing results related to *B. ovis*, other studies producing monoclonal antibodies have already been conducted, in studies related to other species of *Babesia* sp, namely *B. bovis* (Dominguez, et al., 2004), *B. cabbali*, *B. equi* (Bruning, Phipps, Posnett, & Canning, 1997) and *B. bigemina* (Figueiroa, Buening, Kinden, & Green, 1990) where they were recognized, by the monoclonal antibodies produced, proteins with molecular weight similar to the protein recognized in present work, and if the production of monoclonal antibodies against *B. ovis* was achieved, its evaluation in relation to other species would be necessary.

4.5 Flow cytometry to quantify *Babesia ovis*

The present study comprises the first attempt to use flow cytometry (FACS) as a *B. ovis* quantification methodology. FACS is now a widely used as method for analyzing the expression of cell surface and intracellular molecules, characterizing and

defining different cell types in a heterogeneous cell population, assessing the purity of isolated subpopulations and analyzing cell size and volume. The ability to measure fluorescence intensity produced by fluorescent labeled antibodies detecting proteins, or ligands that bind to specific cell-associated molecules, such as propidium iodide binding to DNA, permits its usage in *B. ovis* erythrocyte culture since RBC are non-nucleated. The infected cells are incubated in tubes or microtiter plates with a molecule capable of specifically bind to DNA strands such as SYBR and after analyzed on the flow cytometer (Jang, et al., 2014). This approach has been very useful in determination of parasitemia or confirmation of parasitemia in other organisms.

Herein, a pilot assay was conducted resulting in interesting results, whereas it was possible to measure a parasitemia curve using FACS (Figure 24, 25). However, during these trials, the available fluorescence microscope was damaged, and it was not possible to validate the curve by microscopy. Optimization of this procedure will for sure be very useful and as an example can be used to quickly test susceptibility to new drugs.

4.4. General conclusion and future perspectives

In the present study polyclonal antibodies against *B. ovis* were generated, applying hybridomas production technology. Although not achieving the production of mAbs, such results contribute for the development of screening tests and diagnosis of *B. ovis.*

Besides, the production of anti- *B. ovis* antibodies have important applications, particularly the tick research field where tools are scarce. For example, such antibodies can be used to evaluate and even visualize infection in tick tissues. Depending on the specificity of the antibody (e.g. it can target specific sexual phase proteins) it can be used to differentiate parasite developmental stages, ultimately allowing to follow *B. ovis* life cycle within the tick. In the vertebrate host side, these specific antibodies can be tested in *in vitro* cultures of *B. ovis* to infer about morphological changes, inhibition of RBC invasion and inhibition of growth.

Throughout this study, the *in vitro B. ovis* was maintained to serve as a source of protein extracts and to screen for produced hybridoma supernatants. Continuous maintenance of this culture was initially thought to be also used in anti- *Babesia* drug testing whereas a rapid and simple quantification method rather that microscopy would

greatly aid such trials. Thus, herein the proof of concept for using flow cytometry, as a *B. ovis* quantification method, was also performed. Further validations are still required but such improvement in *B. ovis* detection will for sure be vital in future studies focusing *Babesia* spp..

References

Alberts, B., Johnson, A., & Lewis, J. e. (2002). *Molecular Biology of the Cell.* (4th ed.). New York: Garland Science.

Anaya, J., & Shenfeld, Y. (2013). *Innate imune system in: Autoimmunity: From Bench to Bedside [Internet].* (B. Aristizábal, & Á. González , Eds.) Bogota, Colombia: El Rosario University Press.

Antunes, S., Couto, J., Ferrolho, J., Rodrigues, F., João, N., Santos, A. S., . . . Domingos, A. (2018). *Rhipicephalus bursa* Sialotranscriptomic Response to Blood Feeding and *Babesia ovis* Infection: Identification of Candidate Protective Antigens. *Frontiers in Cellular and Infection Microbiology, 8*.

Bonam, S. R., Partidos, C. D., Halmuthur, S. K., & Muller, S. (2017). An Overview of Novel Adjuvants Designed for Improving Vaccine Efficacy. *Trends in Pharmacological Sciences, 38*(9), 771-793. Retrieved from https://doi.org/10.1016/j.tips.2017.06.002

Brüning, A., Phipps, P., Posnett, E., & Canning, E. U. (1997). Monoclonal antibodies against *Babesia caballi* and *Babesia equi* and their application in serodiagnosis. Veterinary parasitology, 68(1-2), 11–26. https://doi.org/10.1016/s0304-4017(96)01074-6

Cerny, J., & Ilja, S. (2019). *Adaptive innate immunity or innate adaptive immunity?*

Chaplin, D. D. (2010). Overview of the immune response. *Journal of Allergy and Clinical Immunology, 125*(2), S3-S23. Retrieved from https://doi.org/10.1016/j.jaci.2009.12.980

Chauvin, A., Moreau, E., Bonnet, S., & Plantard, O. (2009). *Babesia* and its hosts: adaptation to long-lasting interactions as a way to achieve efficient transmission. *Veterinary Research, 40*(2). doi:10.1051/vetres/2009020

Congdon, R. W., Muth, G. W., & Splittgerber, A. G. (1993). The binding Interaction of Coomassie Blue with Protein. *Analytical Biochemistry, 213*(2), 407-413. doi:10.1006/abio.1993.1439

de Villiers, L., Quan, M., Troskie, M., Jordaan, J. C., & Leisewitz, A. L. (2019). A Comparison Between Manual Count, Flow Cytometry and Quantitative Real-Time Polymerase Chain Reaction as a Means of Determining *Babesia rossi* Parasitaemia in Naturally Infected Dogs. *Acta Parasitologica*. doi:10.2478/s11686-019-00134-9

Dominguez, M., Zabal, O., Wilkowsky, S., Echaide, I., Torioni de Echaide, S., Asenzo, G., Rodríguez, A., Zamorano, P., Farber, M., Suarez, C., & Florin-Christensen, M. (2004). Use of a monoclonal antibody against *Babesia bovis* merozoite surface antigen-2c for the development of a competitive ELISA test. Annals of the New York Academy of Sciences, 1026, 165–170. https://doi.org/10.1196/annals.1307.025

Farber, D., Netea, M., Radbrunch, A., Rajewsky, K., & Zinkernagel, R. (2016). *Immunological memory: lessons from the past and a look to the future*.

Figueroa, J. V., Buening, G. M., Kinden, D. A., & Green, T. J. (1990). Identification of common surface antigens among *Babesia bigemina* isolates by using monoclonal antibodies. Parasitology, 100 Pt 2, 161–175. https://doi.org/10.1017/s0031182000061163

Foltz, I. N., Karow, M., & Wasseman, S. M. (2013). *Evolution and Emergence of Terapeutic Monoclonal Antibodies: What Cardiologist Need to Know*, pp. 2222-2230. doi:10.1161/CIRCULATIONAHA.113.002033

Frank, S. A. (2002). *Immunology and Evolution of Infection Disease.* New Jersey: Princeton University Press.

Funaro, A., Horenstein, A. L., Santoro, P., Armando, G., Gregorini, A., & Malavasi, F. (2000). Monoclonal antibodies and therapy of human cancers. *Biotechnology Advances, 18*(5), 385-401.

Gallagher, S. R. (2006). One-dimensional SDS gel electrophoresis of proteins. In S. R. Gallagher, *Current Protocols in Immunology* (pp. 8.4.1-8.4.37). doi:10.1002/0471142735.im0804s75

Goding, J. W. (1996). *Monoclonal Antibodies: Principles and Practice* (third ed.). Prahran, Australia: Academic Press.

Gough, J. M., Jorgensen, W. K., & Kemp, D. H. (1998). Development of tick gut forms of *Babesia bigemina* in vitro. *The Journal of eukaryotic microbiology*, *45*(3), 298-306.

Hanack, K., Messerschmidt, K., & Listek, M. (2016). Antibodies and Selection of Monoclonal Antibodies. In *Protein Targeting Compounds. Advances in Experimental Medicine and Biology* (pp. 11-22). Springer, Cham. doi:10.1007/978-3-319-32805-8_2

Homer, M. J., Aguilar-Delfin, I., Telford III, S. R., Krause, P. J., & Persing, D. H. (2000). Clinical microbiology reviews. *Babesiosis, 13*(3), pp. 451-469.

Jalovecka, M., Hajdusek, O., Sojka, D., Kopacek, P., & Malandrin, L. (2018). The Complexity of Piroplasms Life Cycles. *Frontiers in Cellular and Infection Microbiology, 8*(248). doi:10.3389/fcimb.2018.00248

Jang, J. W., Kim, J. Y., Yoon, J., Yoon, S. Y., Cho, C. H., Han, E. T., . . . Lim, C. S. (2014). Flow Cytometric Enumeration of Parasitemia in Cultures of *Plasmodium falciparum* Stained with SYBR Green I and CD235A. *Scientific World Journal, 536723*. doi: 10.1155/2014/536723

Jia, N., Zheng, Y.-C., Jiang, J.-F., Jiang, R.-R., Jiang, B.-G., Wei, R., . . . Cao, W.-C. (2018). Human Babesiosis Caused by a *Babesia crassa*–Like Pathogen: A Case Series. *Clinical Infectious Diseases, 67*(7), 1110-1119. Retrieved from https://doi.org/10.1093/cid/ciy212

Klitgaard, J. L., Cljee, V. W., Andersen, P. S., Rasmussen, L. K., Nielsen, L. S., Haurum, J. S., & Bregenholt, S. (2006). Reduced susceptibility of recombinant polyclonal antibodies to inhibitory anti-variable domain antibody responses. *The Journal of immunology*, 177:3782-3790.

Köhler, & Milstein. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity, reprinted in Journal of Immunology. *Journal of immunology*, 495-497.

Kwakkenbos, M. J., Helden, P. M., Beaumont, T., & Spits, H. (2016). Stable long-term cultures of self-renewing B cells and their applications. *Immunological Reviews, 270*(1), 65-77. doi:10.111/imr.12395

Levy, M. G., & Ristic, M. (1980). *Babesia bovis*: Continuous Cultivation in a Microaerophilous Stationary Phase Culture. *Science, 207*, 1218-1220.

Lipman, N. S., Jackson, L. R., Trudel, L. J., & Weis-Garcia, F. (2005). Monoclonal Versus Polyclonal Antibodies: Distinguishing Characteristics, Applications, and Information Resources. *ILAR Journal, 46*(3), 258-268. Retrieved from https://doi.org/10.1093/ilar.46.3.258

Maeda, H., Hatta, T., Alim, M. A., Tsubokawa, D., Mikami, F., Matsubayashi, M., . . . Tanaka, T. (2016). Establishment of a novel tick-*Babesia* experimental infection model. *Scientific Reports, 6*(37039). doi:10.1038/srep37039

Mahmood, T., & Yang, P. C. (2012). Western Blot: Technique, Theory, and Trouble Shooting. *Noth Americal Journal of medical sciencies, 4*(9), 429-434. doi:10.4103/1947-2714.100998

Messerschmidt, K., Hanack, K., & Listek, M. (2016). Antibodies and Selection of Monoclonal Antibodies. Em *Protein Targeting Compounds* (Vol. 917, pp. 11-22). Advances in Experimental Medicine and Biology. doi:10.1007/978-3-319-32805-8_2

Moeko, S., Takeshi, H., Hiroki, M., Tsubokawa, D., Abdul, A. M., Naotoshi, T., . . . Rika, U.-S. (2017). Application of Percoll density gradient centrifugation for separation of *Babesia ovata* infected erytrocytes. *Journal of Protozoology Research, 27*(1-2), 8-12.

Netea, M. G., Schlitzer, A., Placek, K., Joosten, L. A., & Schultze, J. L. (2019). *Innate and Adaptive Immune Memory: an Evolutionary Continuum in the Host's Response to Pathogens*.

Ollila, J., & Vihinem, M. (2005). B cells. *The International Jounal os Biochemistry & Cell Biology*, 523-518. Retrieved from https://doi.org/10.1016/j.biocel.2004.09.007

Pandey, S. (2010). Hybridoma Technology for Production of Monoclonal Antibodies. *International Journal of Pharmaceutical Sciences Review and Research, 1*(2), 88-94.

Parija, S. C., KP, D., & Venugopal, H. (2015). Diagnosis and management of human babesiosis. *Tropical Parasitology, 5*(2), 88-93. doi:10.4103/2229-5070.162489

Pennock, N. D., White, J. T., Cross, E. W., Cheney, E. E., Tamburini, B. A., & Kedl, R. M. (2013). T cell responses: naïve to memory and everything in between. *Advances in Physiology Education, 37*(4), 273-283. doi:10.1152/advan.00066.2013

Phillips, N. E., & Parker, D. C. (1984). *Cross-linking of B lymphocyte Fc gamma receptors and membrane immunoglobulin inhibits anti-immunoglobulin-induced blastogenesis*, pp. 627-632.

Rashid, A., Khan, J. A., Rasheed, K., Maqbool, A., & Iqbal, J. (2010). Prevalence and chemotherapy of babesiosis among Lohi sheep in the Livestock Experiment Station, Qadirabad, Pakistan, and environs. *Journal of Venomous Animals and Toxins including Tropical Diseases, 16*(4).

Rocha, F., & Roncato Duarte, K. M. (2011). Tecnologia de produção de anticorpos monoclonais. *Tecnologia de produção de anticorpos monoclonais*, pp. 183-192.

Rossouw, I., Maritz-Olivier, C., Niemand, J., van Biljon, R., Smit, A., Olivier, N. A., & Birkholtz, L.-M. (2015). Morphological and Molecular Descriptors of the Developmental Cycle of *Babesia divergens* Parasites in Human Erythrocytes. *PLoS Negleted Tropical Diseases, 9*(5), 1-20. doi:10.1371/journal.pntd.0003711

Saad, F., Khan, K., Ali, S., & Akbar, N. (2015). Zoonotic significance and Prophylactic Measure against babesiosis. *International Journal of Current Microbiology and Applied Sciences, 4*(7), 938-953.

Sadeghi Dehkordi, Z., ZaKeri, S., Nabian, S., Bahonar, A., Ghasemi, F., & Noorollahi, F. (2010). Molecular and Biomorphometrical Identification of Ovine Babesiosis. *Iranian Journal Parasitology, 5*(4), 21-30.

Saeed, A. F., Wang, R., Ling, S., & Wang, S. (2017). *Antibody Engineering for Pursuing a Healthier Future, 8*(495), pp. 1-28.

Schnittger, L., Rodriguez, A. E., Florin-Christensen, M., & Morrison, D. A. (2012). Infection, Genetics and Evolution. *Babesia: A world emerging, 12*, pp. 1788-1809.

Sevinc, F., Cao, S., Xuan, X., Sevinc, M., & Ceylan, O. (2015). Identification and Expression of *Babesia ovi*s Secreted Antigen 1 and. *Journal of Clinical Microbiology, 53*(5), 1531-36. doi:10.1128/JCM.03219-14

Shukla, A. A., & Thömmes, J. (2010). *Recent advances in large-scale production of monoclonal antibodies and related proteins, 28*(5), pp. 253-261. doi:10.1016/j.tibtech.2010.02.001

Sonenshine, D. E., & Hynes, W. L. (2008). Molecular characterization and related aspects of the innate immune response in ticks. *Frontiers in bioscience: a journal and virtual library, 13*, 7046-63. doi:10.2741/3209

Tyagi, S., Sharma, P. K., Kumar, N., & Visht, S. (2011). Hybridoma Technique in Pharmaceutical. *International Journal of PharmTech Research, 3*(1), 459-463.

Vannier, E. G., Diuk-Wasser, M. A., Mamoun, C. B., & Krause, P. J. (2015). Babesiosis. *Infectious Disease Clinics of North America, 29*(2), 357-370. Retrieved from https://doi.org/10.1016/j.idc.2015.02.008

Vega, C. A., Buening, G. M., Green, T. J., & Carson, C. A. (1985). *In vitro* cultivation of *Babesia bigemina. American Journal of Veterinary Reshearch, 46*(2), 16-20.

Vega, C. A., Buening, G. M., Rodriguez, S. D., Carson, C. A., & McLaughlin, K. (1985). Cryopreservation of *Babesia bigemina* for *in vitro* cultivation. *American Journal of Veterinary Research, 46*(2), 421-423.

Vidarsson, G., Dekkers, G., & Rispens, T. (2014). IgG subclasses and allotypes: from structure to effector. *Frontiers in Immunology, 5*(520). doi:10.3389/fimmu.2014.00520

Voskuil, J. (2014). *Commercial antibodies and their validation, 232*(3). doi:10.12688/f1000research.4966.2

Wen, Y., Jing, Y., Yang, L., Kang, D., Jiang, P., Li, N., . . . Liu, C. (2019). The regulators of BCR signaling during B cell activation. *Blood Science, 1*(2), 119-129. doi:10.1097/BS9.0000000000000026

Yabsley, M. J., & Shock, B. C. (2013). Natural history of Zoonotic Babesia: Role of wildlife reservoirs. *International Journal for Parasitology: Parasites and Wildlife, 2*, 18-31.